

ENDOCRINOLOGY

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ENDOCRINOLOGY

VOLUME 43

JULY, 1948

NUMBER 1

PERIODICITY IN BODY TEMPERATURE AND HEART RATE

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CHICAGO, ILLINOIS

IT HAS been known for over a century that the human body temperature is not constant, but exhibits regularly recurring fluctuations. A diurnal, or 24-hour, cycle is related to the influence of the daily routine of work, meals, rest, and sleep. A longer cycle in menstruating women has been established more recently, but there are no consistent data on a seasonal or annual temperature rhythm in either sex. The heart rate, which is much more labile than body temperature, has also been found to show a diurnal variation, but figures pertaining to longer-term periodicity are few and contradictory. Furthermore, the relation between body temperature and heart rate, although very definite in cold-blooded animals, as well as in hyperthermia in man, has not been clearly demonstrated in the latter under normal conditions of every-day existence. It is our purpose to present observational data on human body temperatures and heart rates which bear on their diurnal, menstrual and seasonal periodicity; the individual form and constancy of the several cycles; the endogenous and exogenous factors that influence them; and, finally, the close interrelation between the two variables studied.

The tools employed were the ordinary clinical thermometer and the second hand of a watch or clock. Most of the data were obtained by self-observation. The subjects established a routine of existence that they tried to maintain for months, or even years (with occasional breaks for vacations), and those who were temperamentally unfit for regular hours, or wearied of gathering figures, dropped out. For this reason, and also because it takes some weeks to adjust oneself to the life of a "human guinea-pig" the study was intensive, rather than extensive. In all, about two dozen subjects were studied, but this report

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is based on data on ten subjects, six male and four female. Of the male subjects, NK, JN, and HF were middle-aged, and AR, JM, and ML, younger adults; of the females, RN and EH were young adults, and HK and EK, teen-age sisters.

For the diurnal cycle, mouth temperature and radial pulse were determined at two-hourly intervals during the waking hours, and once or twice during sleep, the subject being aroused by an alarm clock set for a definite hour. The thermometer was kept under the tongue for a full five minutes, then read to the nearest $0.05^{\circ}\text{F}.$, and the pulse counted during this period until two successive half-minute counts showed no difference. The observations were made in the sitting or lying position, after at least 15 minutes of rest. Precautions were taken not to expose oneself to outside air during the winter months for half an hour prior to making temperature readings. Likewise, hot and cold liquids were avoided for an equal length of time. By a simple code-marking system, the subjects indicated on their temperature and pulse charts the incidence of meals, taking of tea or coffee; activities, such as work or recreation (card playing, movies, etc); the occurrence and duration of day-time naps, and the time of going to bed and getting up. Meals were timed to follow rather than precede observations. As a longer time unit, the week was found more suitable than the month, since most of the day-to-day variations in routine follow the days of the week. For the menstrual cycle, the day of the onset of menstruation (M) was adopted as the zero point, from which days were counted backward as well as forward. Figures obtained from October to March were applied to winter, while May to August was considered the summer season. For statistical treatment the "t" method of Fisher (1941) was employed, and differences were designated as significant when the probability of their being due to chance was less than one in a hundred ($P < 0.01$). Data for each individual were usually analyzed separately, and attempts were made to bring out not only similarities, but also differences among the several subjects.

I. *The diurnal cycle of body temperature.* The existence of this cycle hardly needed confirmation, but the many thousands of temperature readings we have accumulated permitted us to determine the time of incidence and the constancy of the maxima and minima, as well as the degree of dispersion or concentration of the values pertaining to particular phases of the various cycles. In the several parts of fig. 1 and upper part of fig. 3 are histograms, arranged horizontally, for the frequency with which certain temperature readings occur at different hours in individual subjects. The ranges of readings for almost any hour often exceed the range of the mean diurnal body temperature curve. Yet, distinct modes can usually be made out in frequency distribution curves, formed by the right-side ends of the histograms. The 6 A.M. temperatures of parts A and B of fig. 1, pertaining to one person, show at once the least dispersion and greatest resemblance to

the conventional probability curves. Although the mean maxima of both curves are at 2 P.M. and minima at 2 A.M., many 2 P.M. temperatures are lower than some 2 A.M. values. On the contrary, parts C and D, also of one individual, show a much greater concentration of



FIG. 1. Frequency distribution histograms of mouth temperatures of 6 subjects, all male, as determined for the different hours of the day and night. The vertical temperature scale is the same for all eight parts, the horizontal (number of readings for each temperature value) is to be referred to the length of the bar, next to each letter, which length represents 20. The succession of hours is not to be considered a scale, as these hours are only reference points to the sets of bars above them. A, 3192 temperature readings, 10 per day, of NK, during the summers (mid-May to mid-October) of 1941, 2, and 3; B, 3352 readings for the same subject, during the winter months (October to March) of 1941, 2, 3, and 4; C, 3254 readings, 12 per day, of AR during the summers (June to August) of 1941, 2, and 3; D, 3236 readings for the same subject in the winters (December to February) of 1940, 1, 2, 3, and 4; E, 656 readings, 9 per day, of HF from March to May of 1942; F, 1019 readings, 9 per day, of JH from February to August of 1942; G, 357 readings, 9 per day, of JM from February to April of 1942; and H, 2962 readings, 10 per day, of ML from June 1942 to June 1944. Total number of temperature readings, over 18,000; number of diurnal cycles, over 1,800.

temperature values for every hour of the day and night. In C all the temperatures taken from 11 A.M. to 7 P.M. are higher than all the temperatures from 11 P.M. to 6 A.M. The maxima of both C and D are at 3 P.M., while the minima occur at 3 A.M. giving a 12 hour separation of the two, as in parts A and B. Frequency distribution curves of the incidence of the maxima and minima, not reproduced here, also resemble probability curves, with a greater dispersion of the hours of individual daily maxima than of the daily minima. This results in a

smoother peak and a sharper trough of the diurnal curves, seen even more clearly in the mean diurnal temperature curves for the same subjects in the right-side portions of fig. 2. Part G of fig. 1 resembles parts A to D, even though it is confined to the waking hours and is based on a rather small number of days. The mean maximum is

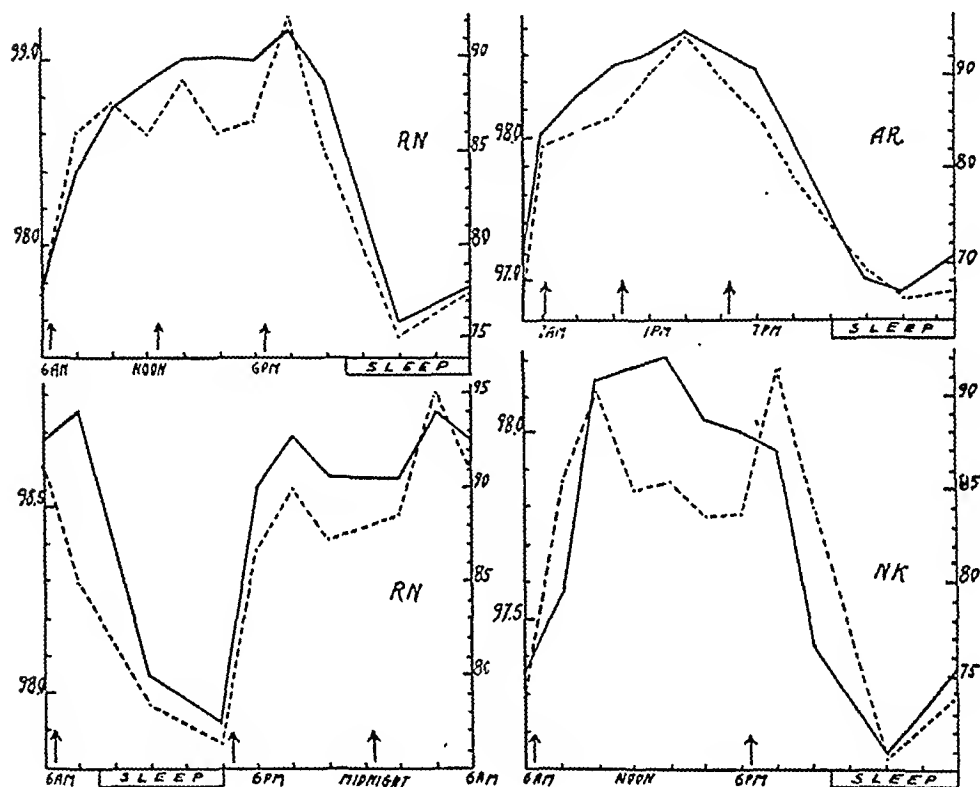


FIG. 2. Mean body temperatures and heart rates for the successive hours of the diurnal cycle. Solid lines, body temperatures, scales on the left of the curves; broken lines, heart rates, scales to the right. Arrows indicate usual meal times. Upper left, data for 168 days, January to June, 1944, 1° corresponding to 10 heart beats; lower left, data for 126 days, June to October, 1944, 1° corresponding to 20 heart beats. The frequency distribution of the temperatures upon which the means in both left-hand figures were based are shown in fig. 3. Upper right, data for 175 days, May to October, 1943, and lower right, for 224 days, May to October, 1943.

98.81° , falling at 2 P.M., and the rise is somewhat steeper than the fall.

A different type of curve is shown in part H of fig. 1. This person's temperature modes are all the same from noon to 4 P.M., although the 2 P.M. mean is the highest, but the maximum daily temperature is reached only at 8 P.M. The minimum is 4 A.M. (not shown in the figure), the maximum-minimum interval being about 8 hours. A similar relationship is found in the upper part of fig. 3, where there is little change in temperature from 2 to 6 P.M. (also pictured in the upper left part of fig. 2), but the maximum is at 8 P.M. and the mini-

mum at 2 A.M., or perhaps a little later. In any case, the maximum-minimum interval is about six hours, resembling part H of fig. 1 in this respect also. We thus have four individuals whose diurnal temperature minima occur at about the same time, during sleep, while the maxima are reached either in the afternoon or in the evening, but are constant for the particular person.

A third type of curve is seen in parts E and F of fig. 1. Instead of

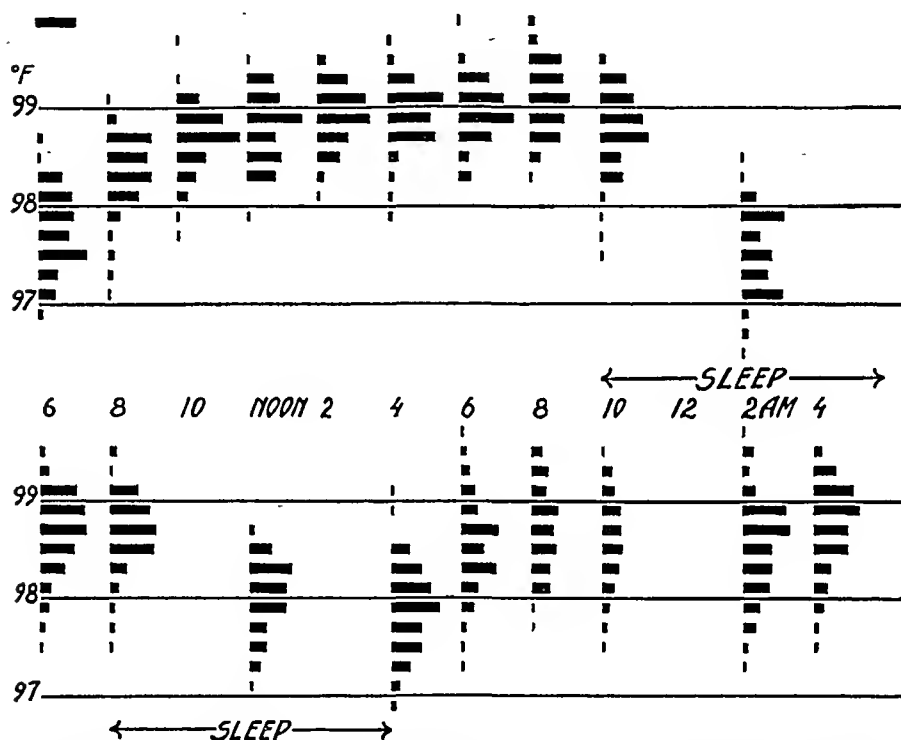


FIG. 3. Frequency distribution histograms of mouth temperatures of RN, female, arranged in the same way as in fig. 1. The mean values of these temperatures are plotted in the solid lines of the left-hand parts of fig. 2.

a peak, there is a plateau, which is quite distinct in E, and almost extreme in F. In E the morning rise is small, and the modes are the same, 98.0° , from 8 A.M. to 8 P.M. The mean temperatures do not vary more than 0.02° from noon to 4 P.M. In F the rise is sharper from 6 A.M. to 10 A.M., but thereafter the mode is the same, 98.6° , till 10 P.M. From noon to 8 P.M., the mean temperatures do not vary by more than 0.07° . This is the only subject whose temperature was the hypothetical 98.6° in about 40% of all the readings made between 10 A.M. and 10 P.M.

II. *The diurnal cycle of heart rate.* Though one of the most labile of physiological values, the heart rate figures arranged in frequency

distribution histograms show the same picture as do body temperatures in figs. 1 and 3, and are therefore omitted. However, the mean diurnal curves of heart rates are usually less smooth than the corresponding temperature curves (fig. 2), and in most subjects the heart rate tends to rise sharply after meals. In the upper left part of fig. 2 there are three such post-prandial rises, while in the lower right part of the same figure, with only two meals per day, there are two sharp heart rate peaks, at 10 A.M. and 8 P.M. In the upper right part, however, a complete parallelism exists between the mean diurnal curves for body temperature and heart rate, every upturn and downturn in one being closely followed in the other. By and large, the heart rates of these, as well as other subjects studied, all show distinct diurnal curves, and by adjusting the scales, these curves can be seen to correspond to the body temperature curves, with a swing of one degree F. involving a change of 10 to 20 heart beats per minute, the exact equivalents varying with the individual and with the daily routine followed. These equivalents are roughly 20 beats in NK, 15 beats in AR, 10 beats in RN under regular routine and 20 beats under a schedule of night work and daytime sleep. Further correlations between the two variables studied will be brought out in considering the endogenous and exogenous influences on the organism.

III. *The effects of changes in daily routine.* A marked shift in the hours devoted to sleep, as well as to work, led to a rapid change in the diurnal temperature and heart rate curve of RN. In a week's time, her mean lows for both variables were at 4 P.M., the end of her sleep period, as contrasted with 2 A.M., the middle of the night under the usual routine (left top and bottom of fig. 2). However, if one examines the distribution of temperature figures for the same subject in fig. 3, one finds little difference between the 2 A.M. and 6 A.M. values of the night sleep, and the noon and 4 P.M. values of the day sleep. More significant is the rapid rise in temperature upon getting up at 4 P.M., compared to a much slower one on a daytime work routine (figs. 2 and 3). The 8 P.M. high, although exceeded slightly by the 4 A.M. and 8 A.M. peaks, still corresponds to the usual 8 P.M. maximum, thus showing a failure to obliterate completely the usual temperature cycle, in spite of a relatively prompt change-over of the diurnal temperature curve to conform to the new routine. Another important difference between the mean temperature curves of RN is the range of 1.57° under usual conditions and one of 0.83° with daytime sleep. Yet, a glance at fig. 3 reveals that the over-all distribution of temperature figures is about the same under the two regimes, about 3° , with only some 2 A.M. temperatures in night sleep falling much below 97° . The difference in the mean curves may be connected with sleep under the shifted routine occurring during the warmest part of the diurnal cycle and wakefulness during the coolest, but the instability of the temperature cycle, as reflected in the lack of distinct temperature modes for 6, 8 and 10 P.M., following daytime sleep, is

also a factor. By contrast, the adaptation of the heart rate cycle to the new conditions is very good, the post-prandial peaks appearing in the proper places (except after the 6:30 A.M. "supper" which was closer than usual to bed-time), the mean diurnal range, 28.8 beats, not differing from the normal range of 27.1 beats.

Smaller shifts in routine, of 3 or 4 hours, lasting from 6 to 10 weeks at a time, were made by AR and NK. Fig. 4 shows a mean weekly "getting up" temperature of 97.2° for AR after arising at 3 A.M. for a number of weeks. Changing the hour of getting up to 6 A.M. raises the basal temperature, with a gradual descent to the previous figures.

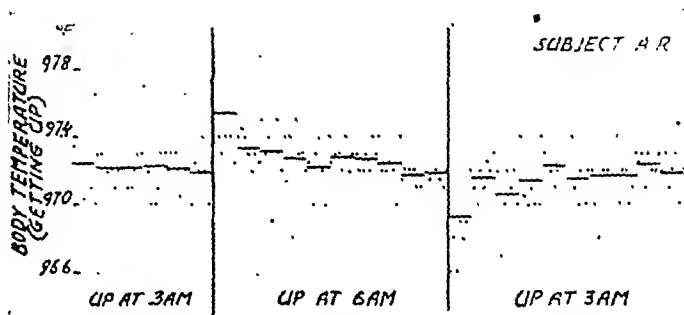


FIG. 4. Basal, or "getting-up," temperatures for 182 consecutive days of AR. Dots represent individual temperature readings, bars, the weekly means.

The same sort of effect, but in the opposite direction, is produced by once again shifting the getting up hour to 3 A.M.

The mean diurnal body temperature range was only slightly increased (from 1.86° to 1.93°) during these shifts for AR, but in the case of NK, during two six-week periods of going to bed between 2 and 3 A.M. and three similar periods of going to bed between 6 and 7 P.M., the mean diurnal temperature range was 1.62° , compared to a mean range of 1.45° , when going to bed at the usual time (about 11 P.M.). The daily maxima and minima were more scattered during shifts than under the normal schedule, with the former tending to be bimodal. On the whole, a better adjustment of the body temperature curve to the new schedule occurred when the onset of sleep was 4 hours later than usual, after 2 A.M., than when it was set 4 hours earlier, after 6 P.M.

IV. *The menstrual cycle.* Data were first gathered on the two sisters (HK and EK) each of whom started taking evening and morning mouth temperatures shortly after she began to menstruate. Fig. 5 shows the frequency distribution of temperatures for successive days of the menstrual cycle, counting back from the first day of menstruation (M) for HK, and a similar distribution, not illustrated, had been obtained for EK. The outstanding feature is the almost complete parallelism between the evening and morning temperatures, the

former a little over one-half degree higher. There is a slow decrease during the first half of the cycle, with a mean low of 98.18° on the evening of day -16 , and a low of 97.59° on the morning of day -15 . Then there is a sharp rise with almost a plateau till two days before the onset of menstruation, when there occurs a sharp dip which is carried over into M day and beyond. The temperature ranges of the mean menstrual curves amounted to 0.74° (evening) and 0.76° (morning) for this subject, but individual swings were usually greater, as ovulation which coincides, or shortly follows, the low point of the

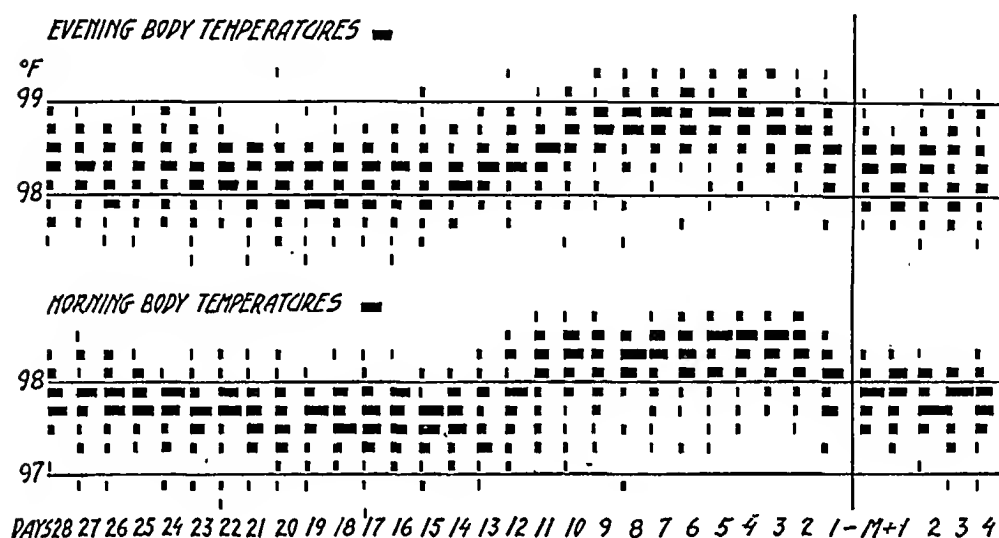


FIG. 5. Frequency distribution histograms of daily mouth temperatures of HK before going to bed at night and immediately upon getting up in the morning, arranged with respect to the first day of menstruation (M). Horizontal bars to the right of each title represent 10 temperature readings. Data start with the second cycle after the onset of menstruation and are based on 34 periods, between Nov. 11, 1941 and Nov. 26 1944. As some of the early menstrual cycles were apparently anovulatory, their exclusion from this series would make the temperature mid-period rises much steeper.

cycle did not always occur on the same day. Thus, an analysis of the 34 cycles whose data were used for fig. 3 shows that the low evening temperature occurred between day -20 and day -13 , with a mean of -15.3 . The individual evening temperature swings of the several cycles varied from 0.70° to 2.15° , the mean range amounting to 1.19° (97.34° – 98.53°). Although the mean morning temperature range was almost exactly the same, 1.20° (97.93° – 99.13°), the latter varied only from 0.60° to 1.65° for individual cycles, and the statistical significance of the mean morning range was higher. The inconstancy of the day of ovulation is also shown by ranges of the mean temperature curves of the other three subjects, as compared with their mean swings: the former are 0.65° , 1.05° , and 0.83° , while the latter are 1.03° , 1.22° , and 1.12° for EK, EH, and RN, respectively. In fig. 6, the composite

basal, or getting-up, temperatures and the corresponding heart rates are shown for these three subjects, as grouped about the low temperature day (O) of the cycle and the first day of menstruation (M). It can be seen that the menstrual temperature curve is mono-cyclic and fairly smooth, while the heart rate curve is definitely di-cyclic and jagged. With respect to O day, the heart rate rises steeply on +1 day and drops almost as much on +2 day, whereas the body temperature continues on its upward course, though in progressively

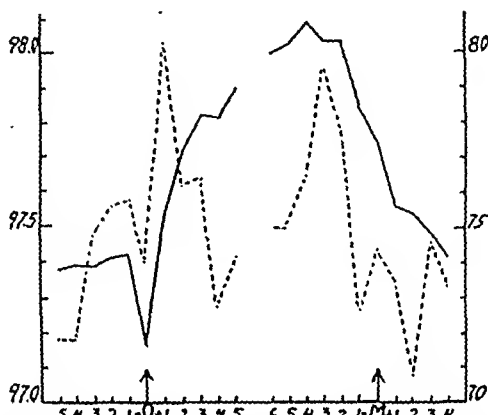


FIG. 6. Composite mean curves of basal mouth temperatures and heart rates of three subjects. The day of low temperature, followed by a steep rise, in each cycle was arbitrarily designated as O day, and the data for — and + days were arranged to straddle this day of supposed ovulation. M day is the first day of menstruation and was therefore fixed for each cycle. Out of a total of 37 cycles, whose values furnished the means for these curves, there were 13 of EK (Feb. 6, 1947 to Jan. 13, 1948), with O day varying from -8 to +13, and a mean of -10.8; 13 of EH (Nov. 12, 1945 to Nov. 13, 1946), with O day varying from -14 to -18, and a mean of -16.3; and 11 of RN (Jan. 1 to Nov. 27, 1944), with O day varying from -11 to -16, and a mean of -13.8. The breaks in the curves are necessitated by the inequality in the intervals separating O and M days in the different subjects, and in the several cycles of each subject.

smaller increments. This combination of heart rate and temperature changes makes it possible to fix the location of O day in the cycle with greater accuracy than by temperature figures alone. A second peak of heart rate, higher or lower than the first, is reached before menstruation, and is followed by a sharp drop, coinciding with, or slightly preceding, the pre-menstrual body temperature fall.

For RN, who took her temperature and heart rate 10 times daily (fig. 3), the mean daily temperatures and heart rates, which we call the temperature and heart rate *levels*, fall into the same curve with respect to O and M days, as do her basal values. In other words, the menstrual curves are the same, whether based on one, two, or ten daily readings. There seems to be an exogenous, probably nervous, influence on the onset of menstruation, as has been pointed out by many writers. Of the two sisters studied, the older, HK, had a mean

menstrual cycle of 32.6 days for the twenty periods, from November, 1941 to September, 1943, before EK began to menstruate. From then until the end of 1945, HK had 26 cycles, whose mean duration was exactly the same, 32.6 days, while EK had 27 cycles, with a mean of 28.7 days. The difference in duration, 3.9 days, was highly significant, and days of onset were often far apart. From January to November of 1946, however, each girl had 10 cycles, with means of 29.1 and 28.7 days respectively, and, in addition, the days of onset of menstruation were, on the average, only 3 days apart.

Menstrual periodicity is abolished during pregnancy and lactation, but each of the latter affects body temperature and heart rate. Al-

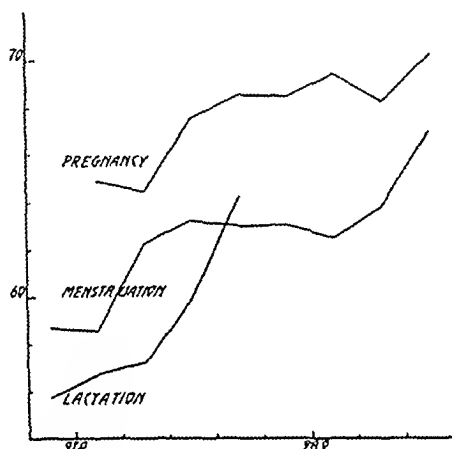


FIG. 7. The relation between mean basal body temperatures and heart rates of EH during pregnancy (Dec. 10, 1944 to June 26, 1945), subsequent period of non-menstruation (July 12 to Nov. 11, 1945), and one year of 13 menstrual cycles (Nov. 12, 1945 to Nov. 12, 1946). Both temperatures and heart rates are higher in pregnancy and lower in lactation than they are during regular menstruation cycles.

though our results are limited to a single pregnancy and subsequent lactation (or, at least, non-menstruation period), they are clear-cut and quite marked. As shown in fig. 7, pregnancy figures for both temperature and heart rate were highest, those for lactation, lowest. That seasonal differences were not responsible was found by dividing the menstruation data into two portions, one to conform seasonally to pregnancy and the other to lactation, and plotting them separately. While the graphs crisscrossed, they were essentially superimposable and just as distinctly below the pregnancy curve and above the lactation one, as is the combined menstruation curve in fig. 7. Further treatment revealed that for a range of 97.4° to 98.4° , comprising 78% of all the pregnancy temperature figures, the mean heart rate was 68.4; for a range of 97.2° to 98.4° , making up 87% of menstrual cycle temperatures, the mean heart rate was 62.9; and for a range of 96.8° to 97.4° , or 76% of the lactation temperatures, the mean heart rate was 56.5. The higher value of the mean heart rate during lactation

when the basal temperatures were 97.6° to 97.8° , as compared to the mean heart rate during the menstruation cycles for the corresponding temperature range, is easily explained by the dicyclic heart rate curve and the monocyclic temperature one during each menstrual cycle, which tends to make the heart rate curve almost a horizontal line between 97.4° and 98.2° .

V. *Seasonal Periodicity.* Among the exogenous influences on body temperature, and thus indirectly on heart rate, there is the seasonal

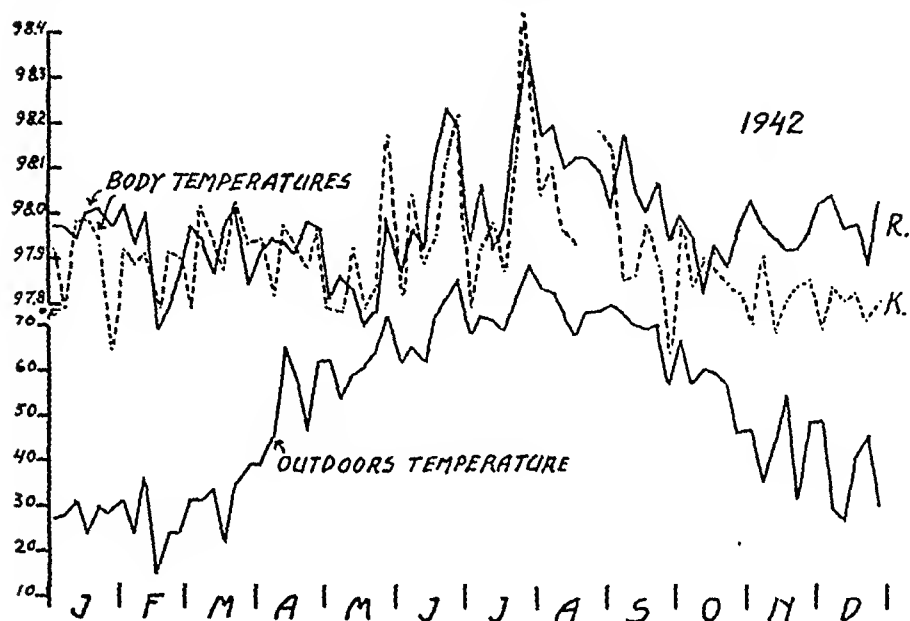


FIG. 8. The variations in successive 5-day body temperature levels of two subjects during 1942 and fluctuations of outdoor temperature levels for corresponding periods.

variation in external temperature. Lest this appear contradictory to the statement that there was essentially no difference in the temperature and heart rate figures for the different seasons in the menstruation curve of fig. 7, it should be pointed out that the latter applied only to "getting-up," or *basal*, values. Indeed, a reference to parts A and C of fig. 1, representing summer body temperatures and parts B and D of the same figure for winter data, will reveal that the 6 A.M. temperatures are the same for both seasons and, as already indicated, have the most prominent modes, 97.2° to 97.4° . for both NK and AR (the same can be said about the basal heart rates, not shown in figure). Otherwise, the occurrence of temperatures over 99.0° is more frequent for both subjects, and the modes are less prominent, in the summer. In the winter, when the inside temperature is kept at a fairly uniform level of about 70° , one lives in an artificial climate, and external temperature variations have no chance to impose their ef-

fects on body temperature under the conditions prescribed for making observations.

To bring out the effects of external temperature we determined the mean of the 10 or 12 observations made at the different hours, which, as mentioned, is designated as the temperature level for the whole 24 hours. Fig. 8 shows the fluctuations of such body temperature levels,

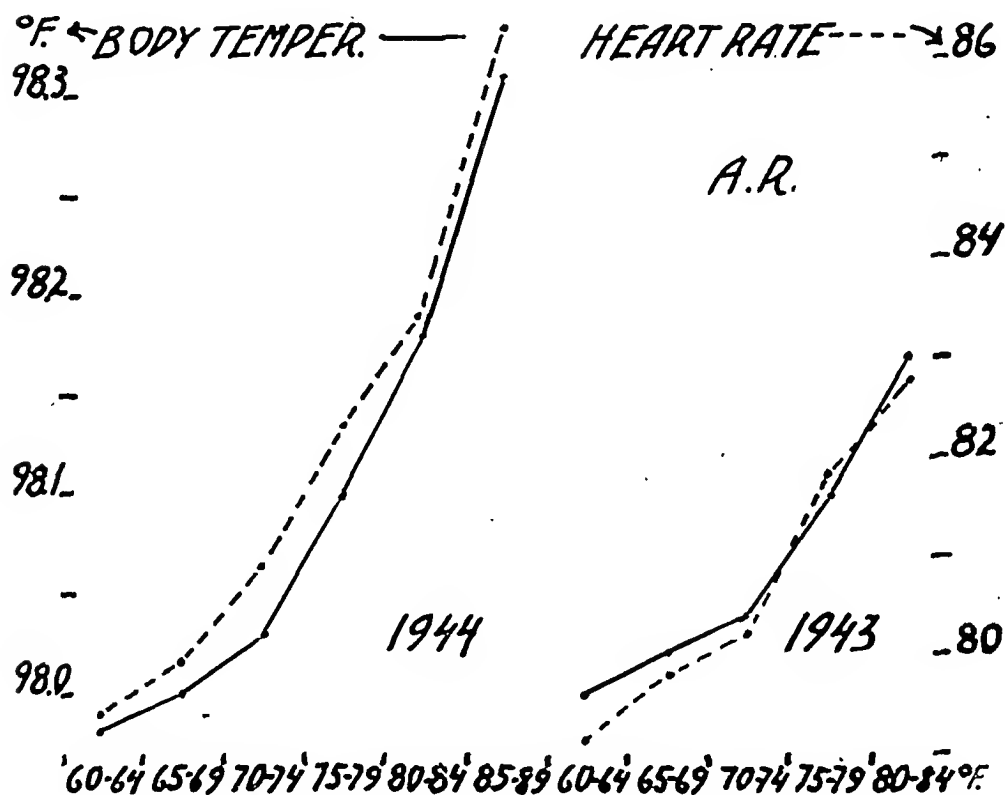


FIG. 9. Mean body temperature and heart rate levels of A.R. for days when the outdoor temperatures fell into the categories shown in the abscissae, for 1943 and 1944. The absence of a 85-89° interval in the 1943 graphs was due to a cooler summer in that year.

means of successive 5 days of each month throughout 1942, for A.R. and N.K. It can be seen that the two body temperature levels rise and fall, independently of each other and of outdoor temperatures during the cold months of the year. Only between the middle of May and the end of September, the period when no artificial heat is supplied, is there a parallellism among the three curves, the warmest five-day stretch of outdoor temperatures corresponding to the highest body temperatures for both subjects. Furthermore, from March to May, when the external temperatures were going up, and after September, when they began to fall, the body temperatures were no different than during January, February, and March. We have similar curves for

other years, as well as for heart rate levels, obtained in the same manner as temperature levels. The relationship between the external temperature and the levels of heart rate and body temperature is shown in fig. 9. The temperature and pulse values for outdoor temperatures from 20° to 60° were omitted as they lie on an essentially horizontal line, both variables beginning to rise, first gradually, and then steeply, only after the outdoor temperatures exceed 60° ("no-heating" season).

Similar curves can be drawn from the data on body temperature and heart rate levels available for NK and RN. These data also afford an opportunity to determine by how many beats the pulse is increased as the body temperature rises with the external temperature. From fig. 9 it can be seen that, for AR, in 1943 a rise in temperature level of 0.17° entails an increase in heart rate of 3.7 beats per minute, corresponding to 21.8 beats per degree F. In 1944, for the same subject, a temperature swing of 0.34° is accompanied by a rise in pulse of 6.9 beats, or 20.3 beats per degree F. Correlation coefficients between the changes in body temperature and those in heart rates are very high, 0.998 and 0.972 for the two years. The week-to-week fluctuations of these values for AR show correlation coefficients of 0.723 and 0.665, both highly significant, for the same two years. The body temperature and heart rate changes for NK, arranged as in fig. 9, give a rise of 8.1 heart beats for 0.48° (correlation coefficient of 0.957), or 16.9 beats per degree F. His week-to-week fluctuations of body temperature and heart rate levels show a correlation coefficient of 0.772. In both subjects, the number of heart beats per degree F. change in body temperature is much higher for lower external, and therefore lower body, temperatures, when the figure may be as high as 35 beats per degree F.

In the mean diurnal body temperature and heart rate curves, shown in fig. 2, on the contrary, AR shows a change of about 15 beats, and NK of 20 beats, per degree F, whereas for RN the number is about 10 beats per degree F. under the usual routine, and 20, when she slept in the daytime. Incidentally, her week-to-week fluctuations of body temperatures and heart rate levels had a correlation coefficient of 0.570 ($P < 0.01$), not as good as those for the male subjects, but still reflecting the effect of the external temperature variations. Again, the corresponding *basal* temperatures and heart rates had a correlation coefficient of 0.370, which was not statistically significant.

VI. *The effects of thyroid.* For a further test of the interrelation between body temperature and heart rate, NK and AR took two grains of desiccated thyroid daily for fourteen days, followed by another 14 days of no thyroid. Basal metabolic rates and body weights were measured at the end of each week. The results were similar for the two subjects, and in fig. 10 can be seen the mean daily levels of body temperatures and for heart of NK, based on 10 successive 28-day periods. The mean metabolic rate went up during the period of thyroid intake, more in the first than in the second week,

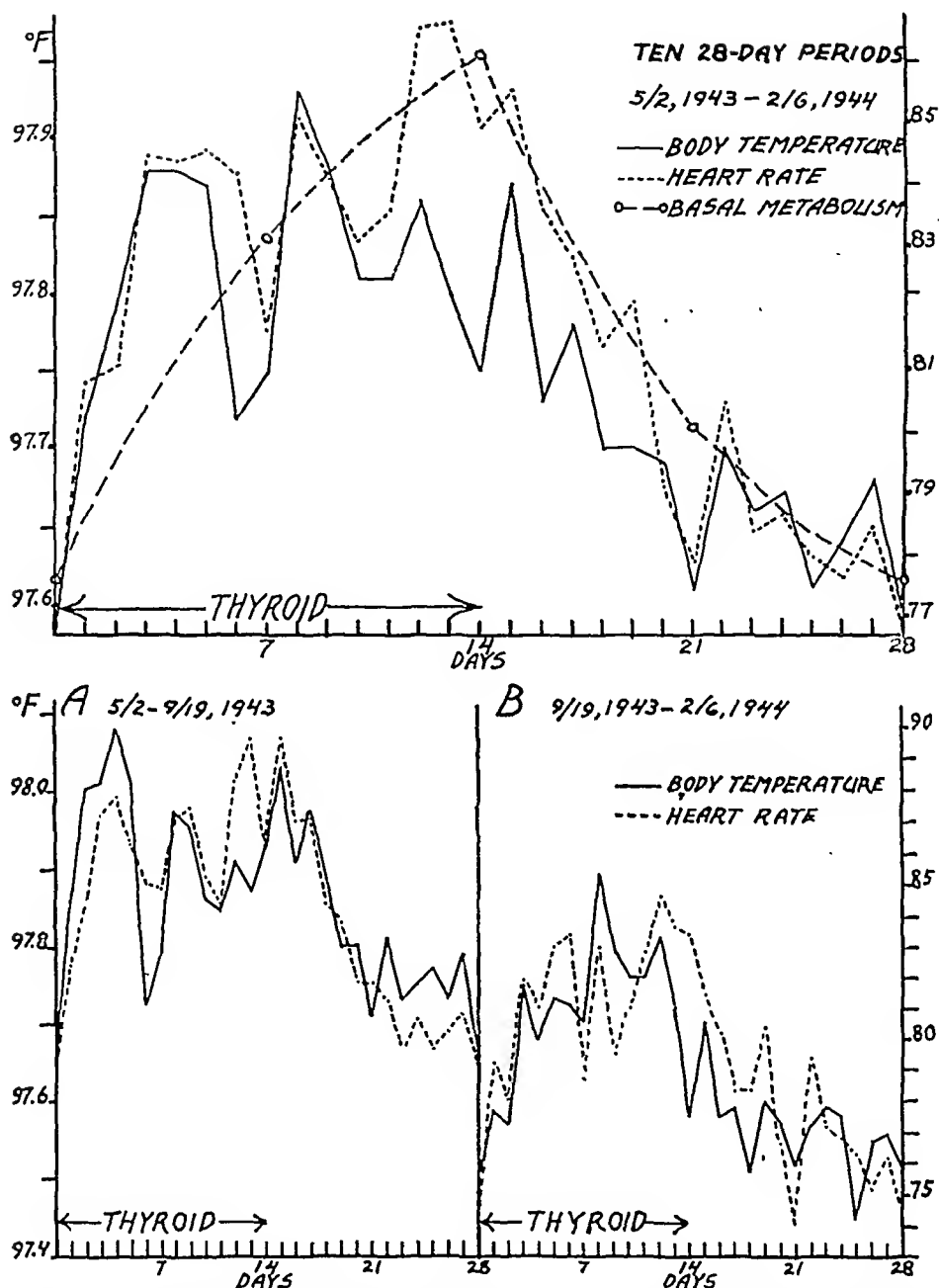


FIG. 10. Upper part, mean daily temperature and heart rate levels of NK, for ten 28-day periods, for the first 14 of which one grain of desiccated thyroid was taken every 12 hours. The mean basal metabolism rates at the end of 7, 14, 21, and 28 days were: 86.2, 89.1, 83.1, and 80.7%, while the corresponding mean body weights were: 151.2, 151.1, 152.6, and 153.6 lbs. Lower part, mean daily levels based on the same data as was upper part, but divided into two 140-day portions (summer and winter.)

while the body weight fell, also mostly during the first week. The reverse occurred during the period of no thyroid, the curve being concave, instead of convex, upward. The body temperatures and heart rates also executed the greater part of their climb during the first week

and showed the sharpest descent during the third. The jaggedness of the curves seems to be related to a weekly periodicity (the body temperature and, to a lesser extent, the heart rate levels for Saturday and Sunday of each week (days 6, 7, 13, 14, 20, 21, 27, and 28) being low, irrespective of thyroid intake). That is perhaps why the basal metabolic rates, determined on successive Sundays, were lower than "normal." The mean day-to-day fluctuations of body temperature and heart rate levels showed a correlation coefficient of 0.916 ($P < 0.01$), and a mean change of 23.4 heart beats per degree F., somewhat greater than reflected in the parallel variations of these two values with changes in outdoors temperature. The seasonal differences, however, are quite marked, as seen from fig. 10 A and B, in which the summer body temperature and heart rate levels are decidedly higher than in the fall-and-winter ones. The correlation coefficient between the mean body temperature and pulse levels for the 10 successive 28-day periods is 0.947; the second was highest with a mean temperature level of 97.96° and a pulse level of 85.4, and the seventh, lowest, with 97.57° and a pulse of 77.5. For a swing of 0.39° the heart rate change was 7.9 beats, or over 20 beats per degree F. The mean difference between each of the 14 days with, and the corresponding days of the 14 days without, thyroid, were highly significant, amounting to, in the summer to 0.19° and 4.5 heart beats, and in the winter, to 0.25° and 5.1 beats, in each case exceeding 20 beats variation per degree F.

VII. *The effect of motion pictures.* As previously reported (Kleitman, 1945) any degree of excitement tends to raise the body temperature above the customary value for the particular hour. Several of our subjects obtained higher than usual temperature figures while attending motion picture shows in the afternoon or evening. Data collected by HK and RN were numerous enough to be treated statistically. The former, on 55 afternoons, spread over a period of more than two years, had mouth temperatures of 99.00° to 100.15° , with a mean of 99.59° . Temperatures taken at about the same hour on 57 afternoons preceding or following "movie" days, ranged from 97.95° to 99.70° , with a mean of 98.66° , or 0.93° lower. Another way to show the effect of motion pictures is to compare the differences between afternoon and basal temperatures on experimental and control days. The mean rises in temperature on 45 "movie" days was 1.69° , while the corresponding value for 54 control days was 0.73° , the differential being 0.96° in favor of the experimental days. Both the 0.93° , from the former treatment, and 0.96° from the latter, are statistically highly significant. RN attended motion picture shows 29 times in the course of two months, viewing 47 feature films, mostly in the evening. At 8 p.m. her control temperatures varied from 98.40° to 99.40° , with a mean of 98.95° , while at the same hour her "movie" temperatures ranged from 99.00° to 99.65° , their mean being 99.42° . The difference between the two means, 0.47° , was highly significant. In this subject,

also, comparing the differences between the mean basal and 8 P.M. temperatures, the rise on control days was 1.15° and on experimental days 1.68° , giving a mean difference of 0.43° , nearly the same as by direct comparison and statistically even more significant.

DISCUSSION

Both body temperature and heart rate are influenced by a variety of endogenous and exogenous factors, some of which serve to establish a number of periodicities in these physiological variables, while others modify or disrupt periodicities thus established. In general, the temperature and pulse cycles are parallel to each other, but in specific situations they may vary in opposite directions. Although the body temperature changes, especially when expressed in terms of absolute values, are extremely small, they are less labile than heart rates and repeat themselves so well as to yield smooth curves, irrespective of the duration of the particular type of cycle. Yet, in a single cycle, even body temperature changes are not easily predictable. The several frequency distribution curves of the diurnal cycle (figs. 1 & 3) illustrate the great dispersion of the readings for almost any hour of the day and night. Under the best of conditions, these distribution histograms arrange themselves into probability curves, with distinct modes, which usually correspond to the means and medians for the same data.

As has been shown some time ago (Kleitman, 1939), the diurnal body temperature curve is established during infancy, with the trough corresponding to the period of sleep. The shape and location of the crest, however, varies with the individual. In some of the subjects there was a peak in the early afternoon, in others, during the evening, and in still others a remarkable semblance to a plateau characterized the diurnal temperature maximum. But even in the latter more than 60% of the temperature readings were different from the prevailing modes. The occupations of AR and JM (fig. 1 C, D, G) who had early afternoon peaks, were the same as those of HF and JH (1 E, F) whose temperatures varied little during the day: they were all supervisors in a cheese factory and were continually moving about. Nor are the two "plateau" curves alike: that for HF is shallow and symmetrical, the small rise from 6 to 10 A.M. being balanced by an equal fall from 6 to 10 P.M., while JH's curve shows an over twice as great a rise in the morning, and a hardly preceptible fall in the evening.

The heart rate follows the diurnal curve closely, except for the postprandial rise in the former, which after the evening meal may appear to go against the rapidly dropping body temperature. Grollman (1929) also observed a rise in pulse rates after meals, but in his subjects they returned to normal within the hour. Boas and Goldschmidt (1932) recall that Haller knew of the postprandial increase in heart rate. They put this increase as 5 to 15 beats per minute in

one hour, with a return to previous level in 2 to 3 hours. The basal, or getting up, temperatures as well as heart rates, are least variable, from day to day in the same individual, and in one person compared to another. Grollman (1929a, 1930), although finding the cardiac output under basal condition more constant than the heart rate, could detect no seasonal variation in the latter. Likewise, Gustafson and Benedict (1928), in 20 female subjects, found no significant seasonal fluctuations in the basal mouth temperatures.

Within the last few years there grew up a literature on the menstrual cycle in body temperature, although Barton (1940), in her excellent historical review, traces early reports to 1870. Little attention was paid to the heart rate variations during the same cycle. King (1914) did notice a general parallelism between basal body temperatures and heart rates during the menstrual cycle, but Rubenstein, (1937) while reporting typical menstrual temperature curves in 10 subjects, disclaimed any "relation between temperature on the one side and blood pressure or resting pulse on the other." It appears that the monocyclic character of the menstrual temperature cycle and the dicyclic one of heart rate cycle, instead of showing no relationship between the two, can be put to advantage in discovering the exact date of ovulation more accurately than by means of body temperature alone. Whereas the basal body temperature rises on consecutive post-ovulative days, the heart rate shows a rise followed by a drop. Using this double check, we found that the position of the ovulation day in the cycle is far from constant, confirming Barton (1942), but that its range and mean value are definite personal characteristics. This is true, even when the duration of the cycle itself undergoes fluctuations from year to year. In our four subjects, the low points in the menstrual temperature curve occurred, on the average, 16.3, 15.3, 13.8, and 10.8 days before the onset of menstruation.

Like Cullis, Oppenheimer, and Ross-Johnson (1922), we found a parallelism between morning and evening body temperature for the menstrual cycle, and the same applies to the temperature levels, or the mean values of several temperatures taken during the diurnal cycle.

Although the pregnancy and lactation data were obtained on one individual, Barton (1940) and others noted an increased body temperature during pregnancy, and Seward and Seward (1936) found that both body temperature and heart rate were higher in this condition.

Mocquot and Palmer (1940) consider axillary temperatures unsatisfactory and insist on rectal temperatures, and Tompkins (1944) also recommends the latter, but Cullis, Oppenheimer and Ross-Johnson (1922) found complete parallelism between rectal and mouth temperatures during the entire menstrual cycle, and Barton (1940) states that "same cyclic phenomena were manifest in oral,

axillary, rectal, vaginal, and uterine temperatures." Following our suggestion (Kleitman, 1944), Davis (1946) found that mouth temperature curves in 500 cases were completely satisfactory.

The exogenous influence of outdoor temperature on both body temperature and heart rate manifests itself, under the conditions of modern indoor existence, only during the portion of the year when no artificial heating is resorted to. Our figures are in agreement with those of Grollman (1930a) whose subjects' heart rate went up from 58 to 64 when the environmental temperature was raised from 68 to 86. It should be noted that the body temperature and heart rate levels of our subjects, and not the basal values, were higher in the non-heating than during the heating season.

Excitement engendered by attending motion picture shows is sufficient to raise the body temperature, and it is possible that listening to certain types of radio broadcasts or reading stimulating books has the same effect.

Changes in the daily routine of work, meals, rest, and sleep can modify the diurnal body temperature and heart rate curves, but there is evidence of a persistence of long established diurnal cycles. The ease with which the change-over is made varies with the individual.

The relation of body temperature or heart rate to the basal metabolic rate of the individual has been a subject of several investigations. Gustafson and Benedict (1928) state that "metabolism tends to be at a low level in the winter and to rise to a higher level during the spring and summer," while Griffith and others (1929) noted a low metabolic rate in the late summer. Rubenstein (1937) first concluded that basal metabolism was independent of body temperature (although his data showed a good correlation between the two), then published curves (1938) showing a fairly complete parallelism between the body temperature and basal metabolic rate during the menstrual cycle in 15 subjects. Williams (1942) found no evidence of any relationship between basal body temperature and metabolic rate, nor of an effect of thyroid on body temperature. Barnes (1942), on the contrary, decided, after studying over 1000 cases, that "sub-normal body temperature is a better index for thyroid therapy than the basal metabolic rate." These discrepancies may be due to the relative constancy of basal temperatures and heart rates. Having made use of the mean daily levels, of both body temperatures and heart rates, we could establish a close relationship between these two variables and the basal metabolic rate. While it is impossible to predict the metabolic rate from body temperature and heart rate data for a given individual, changes in metabolic rate, following thyroid medication, can be followed from day to day by means of body temperature and heart rate levels, and the dose maintained or changed.

[Lastly, the relationship of heart rate changes to those of body temperature can be expressed as the increase in the number of heart beats per minute accompanying a rise in body temperature of one

degree F. This value varies from 10 to 20 beats in the ordinary diurnal, weekly, or seasonal changes in body temperature. It may be as high as 30 or 35, when the body temperatures are low, but for fever Lyon (1927) and others report an increase of 9 to 10 beats (and a range of 5 to 15 beats) for each degree F. of fever.

All our data point to an individual characteristic level of body temperature and heart rate, as well as a personal equation covering the establishment, maintenance, fixity or modifiability of the short or long cycles of these physiological variables. In his *Human Frontier* Williams (1946) states that "each human being has a metabolic pattern which differs in some respects from that of all his fellows." Our findings are in agreement with this conception and are offered as a contribution to the physiology of the individual.

SUMMARY

Mouth temperatures and heart rates fall into parallel diurnal curves adjusted to the routine of existence. Basal, or getting up, values are least variable from day to day and from one season to another.

Basal, evening, or mean daily mouth temperatures exhibit a single wave during each menstrual cycle, while the heart rates show two waves. The crossing of the ascending body temperature and the descending heart rate curves permits a more exact determination of the occurrence of ovulation than body temperature data alone.

Both body temperatures and heart rates are higher in pregnancy and lower in lactation than during the menstrual cycle. During the non-heating season there is a direct relationship between the environmental temperature and both body temperature and heart rate.

Attending motion picture shows raises the body temperature above the level prevailing at the particular time of the day.

Administration of desiccated thyroid raises the body temperature and heart rate in a manner that allows these variables to be used as an index of changes in basal metabolism.

When the body temperature and heart rate vary in a parallel fashion, a rise of one degree F. in the former entails an increase in 10-20 beats per minute in the latter.

ACKNOWLEDGMENTS

We are greatly indebted to the several subjects who went through the painstaking but tedious routine of gathering the reported data by self-observation over long periods of time.

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INFLUENCE OF THE DIET UPON THE NEPHROSCLEROSIS, PERIARTERITIS NODOSA AND CARDIAC LESIONS PRODUCED BY THE "ENDOCRINE KIDNEY"

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EARLIER observations had shown that nephrosclerosis, periarteritis nodosa and myocardial lesions resembling rheumatic carditis can be produced in rats (especially after unilateral nephrectomy) by continuous exposure to various types of stress or treatment with either desoxycorticosterone or corticotrophic anterior-pituitary preparations. It was noted, however, that these pathologic changes are caused by the above agents only if the animals are kept on diets rich in sodium chloride; conversely, diets poor in NaCl (as well as those resulting in a loss of sodium because of added NH_4Cl) protected our experimental animals against the above-mentioned changes normally produced by stress, desoxycorticosterone or anterior-pituitary preparations.

A high protein content of the food failed to influence the renal and cardiovascular lesions caused by desoxycorticosterone acetate, but aggravated those caused by stress or crude anterior-pituitary extracts. (For literature see: Selye, 1946a; Selye, 1947a; Selye, 1947b.)

More recently we found (Selye and Stone, 1946) that with a special surgical procedure it is possible to reduce the blood pressure in the left kidney of the rat to the level of the colloid-osmotic pressure of the blood. Thus filtration pressure is completely abolished and hence the production of urine ceases. At the same time the kidney transforms itself into a massive epithelial organ whose functions are apparently purely endocrine. Bearers of such "endocrine kidneys" acquire a particularly pronounced, and rapidly fatal, hypertension. At the same time they develop malignant nephrosclerosis in the contralateral (urine-producing) kidney, periarteritis nodosa (especially of the mesenteric and pancreatic vessels) and myocarditic changes, similar to those seen in rheumatic fever. Thus, bearers of an endocrine kidney exhibit lesions essentially similar to those which we had previously produced by stress, anterior-pituitary extracts or desoxycorticosterone.

Preliminary observations suggested that the morphologic changes in the cardiovascular system and kidney as well as the hypertension produced by the "endocrine kidney" remain uninfluenced by the protein and sodium concentration of the diet (Selye, 1947c; Selye, 1948). This finding was rather unexpected since these same manifestations can be prevented so readily by such dietary measures if the changes are elicited by stress, desoxycorticosterone or crude pituitary extracts.

It will be recalled, furthermore, that a number of earlier investigators claimed that protein (Verney and Vogt, 1938; Handler and Bernheim, 1948) or sodium (Verney and Vogt, 1938; Hantschmann, 1931; Grollman and Harrison, 1945 and Grollman, 1945) poor diets inhibit the development of hypertensive disease in animals subjected to partial constriction of the renal artery or other interventions suitable for the production of renal hypertension. Others, however, obtained inconclusive results (MacLachlan and Taylor, 1940; Philipsborn et al, 1941 and Guerrant et al., 1943). The rice and fruit-juice diet advocated by Kempner (1945) which is poor both in protein and in sodium, also proved to be effective in reducing the blood pressure in animals suffering from experimental renal hypertension (Grollman and Harrison, 1945).

In view of these contradictory data, we decided to re-examine this problem systematically on a large number of animals, using the endocrine kidney technic as a means of producing acute, malignant, renal hypertension. Rats in which endocrine transformation of the kidney has been obtained by our surgical procedure appeared to be singularly well suited for this type of experimentation. Results obtained in the rat are directly comparable with our earlier work concerning the influence of dietary factors upon hypertensive disease caused by stress, desoxycorticosterone or anterior-pituitary extracts in this species. Furthermore, the endocrine kidney technic has the advantage of producing a uniform degree of renal hypertension, since excessive constriction of the artery causes necrosis, while insufficient constriction, which fails to inhibit urine formation, results in hydronephrosis and hence destroys the left kidney (whose ureter is ligated). Either of these experimental errors is readily detectable at autopsy and only bearers of perfect endocrine kidneys were used for the evaluation of our data.

EXPERIMENTAL

In the present series, we employed female albino rats weighing 130-150 gm.; they were killed on the 11th day after the "endocrine kidney operation." The latter was performed according to the technic of Selye and Stone (1946) and Selye (1946c), using the modification in which the ureter of the endocrine (left) kidney is ligated.

All animals were fed a synthetic basic diet in which only the protein (casein) and sodium (NaCl) concentration was varied. Groups

ENDOCRINE KIDNEY EFFECTS

July, 1948

I-VI were placed on a low protein diet, which contained 10% casein and was made up as follows:

<i>Basic Diet</i>	
	(gm. per 100 gm. of diet)
Casein	10
Cornstarch	83
Fat (Mazola)	1
Cod liver oil	1
CellufLOUR	1
Mineral mixture (see below)	4
Supplements (see below)	<1

Groups VI-XII were kept on essentially the same diet but containing 30% casein; correspondingly the carbohydrate (cornstarch) concentration was diminished from 83 to 63%.

In both these diets the mineral mixture was essentially identical with that described by Steenbock and Nelson (1923).

<i>Mineral Mixture</i>	
	(parts used in making the mixture of which diet contains 4%)
NaCl	23.4
MgSO ₄ ·7H ₂ O	24.6
Na ₂ HPO ₄	14.2
K ₂ HPO ₄	69.6
CaHPO ₄ ·2H ₂ O	69.8
Ca. Lactate. 5H ₂ O	15.4
Ferric Citrate	1.2
KI	.16

The vitamin supplements employed were as follows:

<i>Vitamin Supplements</i>	
	(mg. per 100 gm. of diet)
Thiamine chloride	0.8
Riboflavin	0.8
Pyridoxine	0.8
Ca. pantothenate	1.5
Nicotinic acid	1.5
Choline chloride	400.0
Inositol	100.0
p-amino-benzoic acid	30.0

The minerals were simply added to the other dry components of the diet, while the vitamins were first taken up in a little water and then thoroughly mixed with the other dietary constituents, thus sufficiently moistening the dry material to give it the consistency neces-

sary to prepare small food balls, which were placed into the animals' cages.

In groups IV, V, VI, X, XI and XII, excess of sodium was administered by adding 4% NaCl instead of a corresponding amount of corn starch.

In group XIII we wished to examine the effect of simultaneous complete deprivation in Na and Cl on a low-protein diet. Here, the NaCl was eliminated in the mineral mixture and K_2HPO_4 was substituted for Na_2HPO_4 . While in all other groups the animals were given tap water to drink, in group XIII only distilled water was allowed in order to eliminate any NaCl intake through the drinking fluid. As a further precaution, the animals of group XIII were placed on the NaCl free ration 10 days before the endocrine kidney operation, to insure that their Na-balance would be negative before the experiment commenced.

EXPERIMENTAL OBSERVATIONS

The results of our observations are summarized in Table I.

The "*survival ratio*" as listed in the fourth column gives the number of animals used for the compilation of these data (all animals bearing imperfect endocrine kidneys were discarded) and (in bold type) the number that survived until the 11th day. It will be seen that all intact controls (Groups I, IV, VII and X) survived. Another type of control consisted in performing the endocrine kidney operation but following this—during the same intervention—by the removal of the left kidney. Controls of this type were deemed necessary in order to eliminate the possible influence of the partial constriction of the aorta, quite apart from any "endocrine transformation" of renal tissue. It will be noted that these controls (Groups III, VI, IX and XII) also survived to the end of the experiment with the sole exception of one animal in group VI. On the other hand, the mortality during the 11 day observation period was very high in all groups in which an endocrine kidney was present (Groups II, V, VIII, XI and XIII). The diet did not appear to exert any very significant influence upon the survival rate of animals possessing an endocrine kidney. Essentially the same is true of the loss of body weight which was manifest in all these groups while the intact controls continued to grow and those in which the endocrine kidney was immediately removed after the operation showed only an insignificant loss of weight, if any.

The weight of the *left kidney* is expressed in mg. as well as in mg./100 cm.² of body surface. The latter method of expression gives perhaps a more correct picture of the amount of kidney tissue available in proportion to the metabolic needs of the organism. It will be noted that the weight of the kidney decreased considerably as a result of the "endocrine transformation" and this involution appears to have

TABLE I
INFLUENCE OF THE DIET UPON THE MORPHOLOGIC LESIONS INDUCED BY THE "ENDOCRINE KIDNEY"
(Means and Standard Errors)

Group	Treatment	Diet	Survival ratio		Body weight		Left kidney		Right kidney		Heart		Pancreas- teritis nodosa (in %)	Adrenals (mg.)
					Initial gm.	Final gm.	Mg.	Mg./100 sq. cm. body surface	Mg.	Mg./100 sq. cm. body surface	% of body weight	Myo- carditis (in %)		
I	Intact Controls	10% Casein	8:8		130 ± 3	138 ± 4	404 ± 19	191 ± 7	485 ± 19	200 ± 7	0.35 ± 0.01	0	0	27 ± 2
II	Endocrine Kidney	10% Casein	22:15		142 ± 2	97 ± 3	248 ± 11	131 ± 7	637 ± 20	338 ± 12	0.53 ± 0.01	50	70	44 ± 2
III	Endocrine Kidney Removed	10% Casein	15:15		145 ± 2	140 ± 3	—	—	708 ± 23	294 ± 8	0.33 ± 0.01	0	0	39 ± 1
IV	Intact Controls	10% Casein 4% NaCl	7:7		130 ± 2	130 ± 3	520 ± 24	210 ± 8	548 ± 28	227 ± 9	0.35 ± 0.01	0	0	28 ± 2
V	Endocrine Kidney	10% Casein 4% NaCl	19:8		146 ± 4	98 ± 2	257 ± 23	143 ± 14	694 ± 51	352 ± 28	0.54 ± 0.02	60	48	56 ± 7
VI	Endocrine Kidney Removed	10% Casein 4% NaCl	10:9		145 ± 4	135 ± 4	—	—	702 ± 17	295 ± 6	0.36 ± 0.02	0	0	41 ± 3
VII	Intact Controls	30% Casein	8:8		130 ± 2	145 ± 4	579 ± 26	231 ± 8	603 ± 29	240 ± 9	0.36 ± 0.01	0	0	33 ± 1
VIII	Endocrine Kidney	30% Casein	27:14		146 ± 2	112 ± 5	271 ± 16	129 ± 6	775 ± 33	369 ± 13	0.50 ± 0.02	70	63	43 ± 2
IX	Endocrine Kidney Removed	30% Casein	11:11		147 ± 3	147 ± 3	—	—	842 ± 25	335 ± 8	0.33 ± 0.01	0	0	45 ± 3
X	Intact Controls	30% Casein 4% NaCl	8:8		130 ± 2	148 ± 3	646 ± 18	254 ± 6	670 ± 17	266 ± 5	0.35 ± 0.01	0	0	34 ± 1
XI	Endocrine Kidney	30% Casein 4% NaCl	17:8		144 ± 2	122 ± 5	308 ± 31	138 ± 13	823 ± 21	369 ± 8	0.48 ± 0.02	71	60	40 ± 3
XII	Endocrine Kidney Removed	30% Casein 4% NaCl	10:10		143 ± 3	140 ± 3	—	—	906 ± 38	370 ± 14	0.36 ± 0.02	0	0	48 ± 2
XIII	Endocrine Kidney	10% Casein Na and Cl free	9:8		131 ± 2	117 ± 6	341 ± 15	158 ± 7	971 ± 22	312 ± 17	0.50 ± 0.02	52	54	44 ± 3

been entirely independent of the dietary casein and NaCl concentration. On the other hand, among the intact controls, the mean kidney weight was lowest in group I (low casein, low NaCl diet), somewhat higher in group IV (low casein, high NaCl diet), still higher in group VII (high casein, low NaCl diet) and highest in group X (high casein, high NaCl diet). Though not specifically mentioned in the table, all the endocrine kidneys exhibited essentially the same histologic structure, the most characteristic features of which were involution of the glomeruli, loss of eosinophil granules and disappearance of the brush border in the proximal convoluted tubule cells, obliteration of the tubular lumina by proliferating lining epithelium and marked proliferative changes in various parts of the proximal convoluted segment, especially in the distal part, the so-called spiral segment. Nephrosclerosis or hyalinization of blood vessels was never observed in endocrine kidneys, even when the contralateral kidney showed most pronounced changes of this type.

The mean weight of the *right kidney*, among the intact control animals, ran roughly parallel to that of the left. The significance of this kidney-weight increasing effect of dietary protein and NaCl is further supported by the weights of the right kidneys in the animals whose left kidney had been removed at the beginning of the experiment (Groups III, VI, IX and XII). This exhibits essentially the same rise with increasing protein and NaCl concentrations in the diet, the only difference being that here the right kidney is much larger because of the compensatory hypertrophy produced by ablation of the contralateral organ.

The weight of the right kidney in those animals which bear an endocrine kidney on the other side (Groups II, IV, VIII and XI) is less markedly influenced by dietary factors. Histologic examination of these kidneys shows them to be affected by nephrosclerosis with dilatation of tubules, deposition of hyalin material (casts) in their lumina and hyalinization of the blood vessels. Therefore most of the enlargement cannot be regarded as a true compensatory hypertrophy but is more comparable to the increase in renal weight seen in the acute stage of other types of nephrosclerosis, for instance, that produced by desoxycorticosterone or anterior-pituitary preparations.

The nephrosclerosis is listed as a percentage of the theoretic maximum which could have been obtained. The diagnosis was made by one of us upon inspection of histologic slides labelled only with code numbers whose significance he did not know. This was deemed necessary to assure absolute objectivity of this, otherwise rather subjective, type of diagnosis. The degree of nephrosclerosis was expressed in a scale ranging from 0 to + + +; hence the theoretic maximum of + + + in all animals was considered as equivalent to 100%. It will be noted that when so expressed, the degree of nephrosclerosis was approximately 50% in all groups including even group XIII in which a casein-poor diet was given in the complete absence of NaCl.

The *heart* weight is expressed both in mg. and as a percentage of

body weight. It will be noted that according to either manner of expression, the heart weight is increased in the endocrine kidney bearing rats (Groups II, V, VIII, XI, XIII) as compared to the corresponding intact controls or those in which the endocrine kidney was removed. The degree of this cardiac hypertrophy was not significantly influenced by the diet. The heart weight is a fairly accurate expression of the mean blood pressure during the entire experimental period. Indeed some investigators believe the heart weight to be a more accurate index of hypertension than the occasional actual measurement of the blood pressure at intervals throughout the experiment, because it is less likely to be influenced by sudden transient variations. No systematic blood-pressure measurements were made in groups I–XII of this series, yet careful determinations of this type (performed under light ether or nembutal anesthesia by the direct method) revealed that in group XIII the endocrine kidney caused marked hypertension (up to 180 mm. of mercury mean pressure in some cases). This shows that renal-pressor material can be produced, and when present can raise the blood pressure in the complete absence of NaCl even on low-protein diets.

The myocarditic lesions produced by the endocrine kidney in groups II, V, VIII, XI and XIII were likewise determined as a percentage of the maximum total, on the basis of histologic examination under conditions identical with those described above for the kidney. It will be noted that the diet also failed to influence the intensity of the histologically demonstrable cardiac damage caused by the endocrine kidney.

Periarteritis nodosa was noted on histologic sections in the pancreas since the vessels of this organ are especially subject to periarteritic lesions. The diet did not appear to influence the intensity of the hyalinization and granuloma formation.

The *adrenals* were greatly enlarged in all endocrine-kidney bearing rats, again without any manifest relationship to the diet consumed. It will be recalled that the corticotrophic effect of stress and of impure anterior-pituitary extracts is greatly dependent upon the dietary protein intake although independent of the sodium content of the food (Selye, 1947b). It has been assumed in previous publications from our laboratory that a certain amount of protein catabolism is essential for the maximal production of adreno-corticotrophic hormone; perhaps in the present experimental series the protein-catabolic effect of the renal pressor material was sufficient to furnish optimal amounts of protein catabolites, even when dietary protein intake was low.

DISCUSSION

Our earlier observations had shown that ingestion of excessive amounts of NaCl increases, while sodium deprivation (due to a diminished Na intake or a forced Na-loss through the urine, e.g., after NH_4Cl medication) diminishes the hypertensive and nephrosclerotic activity of desoxycorticosterone (Selye and Stone, 1943; Selye and

Hall, 1943a; Selye and Hall, 1943b; Selye et al., 1944). It has also been demonstrated that the production of nephrosclerosis, vascular changes and hypertension by anterior-pituitary preparations is likewise inhibited by sodium depletion (Hall et al. 1945). The adrenotrophic effect of crude anterior-pituitary extracts, on the other hand, remains uninfluenced by sodium but is greatly diminished in animals maintained on low-protein diets. At the same time protein depletion also prevents the production of nephrosclerosis, hypertension and cardiovascular lesions by anterior-pituitary extracts (Berman et al., 1945; Selye, 1946b; Hay et al., in press). On the other hand the nephrosclerotic and hypertensive actions of desoxycorticosterone remain uninfluenced by dietary protein (Prado et al., 1947).

From all these observations it appears that the corticotrophic activity of crude anterior-pituitary extracts (and probably also the corticotrophin production under the influence of stress) are dependent upon the protein intake but independent of the sodium intake; conversely, the action of corticoids such as desoxycorticosterone is dependent upon the availability of sodium but independent of protein.

The present experimental series clearly shows that the hypertension as well as the accompanying cardiovascular and renal changes produced by the endocrine kidney are independent of both the protein and the sodium content of the diet.

As mentioned in the introduction to this paper, pertinent observations of earlier investigators—who studied the effect of the protein and sodium concentration in the diet upon the development of renal hypertension—were rather inconclusive. It must be kept in mind that none of these workers observed animals in which the “endocrine transformation” of the kidney was as complete as in our rats. We cannot give an explanation for the apparently positive results of some investigators but feel that the data presented in this publication clearly indicate that, under the experimental conditions used by us, renal hypertension develops irrespective of the dietary protein and sodium. It will be recalled that the “endocrine kidney technic” suddenly causes complete endocrine transformation of the entire renal parenchyme; it is possible that in the face of such sudden maximal transformation, dietary constituents cannot further increase the production of renal pressor substances. Yet, in the event of less drastic surgical interventions, nutritional factors may play a rôle, perhaps by increasing the production of hypophyseal and corticoid hormones or by augmenting the “endocrine transformation” of individual nephrons under the influence of corticoids.

SUMMARY

Experiments on the rat indicate that the production—by the “endocrine kidney” technic—of hypertension and its morphologic accompaniments (nephrosclerosis, cardiac hypertrophy, myocarditis,

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periarteritis nodosa) remain uninfluenced by the protein and NaCl concentration of the diet.

These findings are contrasted with the marked dependence upon sodium of the production of these same lesions by desoxycorticosterone or anterior-pituitary extracts.

High protein diets do not influence the production of hypertensive disease by desoxycorticosterone but they markedly increase this same effect of crude anterior-pituitary extracts.

It is concluded that protein increases the corticotrophic action and sodium augments the effect of mineralo-corticoids. Anterior-pituitary hormones stimulate corticoid production, while the corticoids cause nephrosclerosis and the formation of "endocrine nephrons." The diet influences the "endocrine transformation" of kidney tissue under the influence of such hormonal stimuli but—as shown by the present experimental series—the secretion and action of the renal pressor hormones themselves remain uninfluenced by the sodium and protein intake.

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ADRENAL AUTOTRANSPLANTS WITH HEPATIC PORTAL DRAINAGE IN THE RAT

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IT HAS been known for many years that the liver can inactivate estrogenic hormone or impair the function of ovarian tissue. It has, likewise, been found that epinephrine is destroyed in the liver since injections of it into the splenic vein produce about a fourth the rise in blood pressure that can normally be expected (Philpot and Cantoni, 1941).

Progesterone, on the other hand, is not inactivated by liver pulp "in vitro" (Engel, 1944). There are also indications that the function of cortical tissue of the adrenal is not impaired by the liver since Eversole, Edelmann and Gaunt (1940) succeeded in maintaining the lives of two of ten rats with adrenal autografts in the liver. Failure in eight of their experiments may have been a matter of technique or the liver may have been antagonistic toward hypertrophy of the tissue of the adrenal.

More recently Vogt (1943) has claimed that the body disposes of released cortical hormone of the mammalian suprarenal at great speed since no detectable amount of the hormone was in the blood from the right side of the heart or from the femoral artery in the dog and cat. The result was the same in the eviscerated animal and, according to him, if the liver played a predominant role in the inactivation of the hormone, the exclusion of the liver from the circulation would lead to the accumulation of cortical substance in the general circulation. No hormone was found in the right atrium and femoral artery by Vogt and, therefore, little existed to be inactivated by the liver. Since the cortical substance encountered few tissues in going from the adrenal to the right atrium and these tissues disposed of most of the hormone the liver might be a great inactivator of cortical substance if quantities of the hormone reached this organ.

The purpose of this investigation has been to learn: (1) if adrenal transplants in the liver or spleen will maintain the life of the rat as well as autografts in the kidney; (2) how much of the adrenal cortex must be transplanted for survival of the animal after bilateral adrenalectomy.

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METHODS

In these experiments rats of the Long-Evans and Wistar strains were used. Since hair growth in the rat is accelerated in the absence of cortical material (Butcher, 1937) (Ralli and Graef, 1943), normal hair growth and the survival of animals were used as indicators of a sufficient supply of cortical hormone in the body. Operations were made soon after weaning or on the twenty-second day of life.

Both adrenals were removed via the dorsal approach and then either an entire adrenal or a portion of one adrenal was autotransplanted into the liver, spleen or kidney. A portion of an adrenal was autotransplanted to compare the inactivating power of the tissues of the kidney and of the liver on the cortical hormone. If the inactivating power of the liver was greater than that of the kidney, more cortical materials would have to be present to maintain life. When a half or a fourth of an adrenal was used as a transplant, an adrenal was cut as nearly as possible into such fractions under the stereoscopic microscope. Transplants were made easily under the capsule of the kidney as described in a previous paper (Butcher, 1937). For holding an autograft of an entire adrenal to the spleen, a fine thread was run through the capsule of the adrenal and then the thread was tied around the spleen. Autografts were anchored and retained with difficulty in small slits in the liver and spleen.

One per cent salt solution served as drinking water for 4 days after the operation and thereafter, only tap water was available. Feed consisted entirely of Purina dog chow.

Animals were sacrificed at intervals following the operations. The autografts were recovered; fixed in Bouin's; stained in hematoxylin and eosin; and then studied. When the animals were sacrificed, the regions around the kidneys were carefully surveyed for accessory cortical material and this tissue was fixed and studied histologically. Animals possessing accessory cortical bodies were not included in this study. Autografts were also recovered as often as possible from animals dying of cortical insufficiency.

AUTOGRAFTS OF ONE-FOURTH OF AN ADRENAL

Twelve of 16 animals receiving autografts of one-fourth of an adrenal in the liver died within 14 days after the operation. On histological examination, the grafts of these animals were found isolated by a thick capsule. The transplants often contained small islands of regenerated tissue immediately internal to the capsule and necrotic masses of cellular debris and pigmented degenerating cells were found centrally. The small islands which apparently originated from the capsule were incapable of supporting the animal.

The other four animals were sacrificed at 35, 70, 100 and 140 days after the operation. One had a litter of six when the graft was 56 days old and the puppies were guarded with great care. They all died, however, within 10 days because of the inadequate milk supply of the mother. Histological examination of the adrenal grafts of these four sacrificed rats showed that they were surrounded with a thin capsule. The grafts were large and were composed of a very vascular glomerulosa and fasciculata but no reticularis was present.

Animals with autografts of one-fourth of an adrenal in the kidney survived no better than those with similar autografts in the liver. Six of the eight animals receiving such grafts died within 12 days after the transplantation. The autografts which were recovered from them were composed of areas of regenerated cortical cells beneath a thick capsule. Two other animals with one-fourth autografts gained weight and were sacrificed 35 and 70 days after the operation. The large grafts from these two animals had a thin capsule and most of the cells were glomerular in character.

Hair growth was accelerated in practically all animals which received an autograft of one-fourth of an adrenal and which died within 12 days after the transplantation.

AUTOGRAFTS OF ONE-HALF OF AN ADRENAL

Four of the ten animals receiving one-half of an adrenal in the liver died within 12 days after the operation. Regenerated islands of cortical tissue were often found next to the capsule in the grafts recovered from the dead animals. The bulk of the transplants, however, consisted of pigmented degenerating cells.

The six other animals receiving one-half adrenal in the liver survived and were sacrificed 48, 70, 96 and 102 days after the transplantation. The recovered grafts were very large and were surrounded by a thin capsule which was continuous with septa extending throughout the gland. Most of the graft consisted of glomerulosa and fasciculata regions. In some areas which were not always centrally located the cells were arranged in a reticular pattern. One rat had a litter 80 days after the operation but the puppies died soon after birth.

Autografts of one-half adrenal in the kidney were equally as successful as in the liver. One animal died on the fifteenth day and two others on the eighteenth day after the operation. The death of one animal 26 days following grafting was surprising since the recovered graft showed several large regenerated islands. These islands were composed of closely packed cells and were separated by septa. They had not reached the differentiation in zonation attained by most grafts in surviving animals. Other animals gained weight and were sacrificed 37, 75 and 140 days after grafting. Nine of this group of 13 animals with grafts in the kidney survived longer than 30 days.

Many of the rats receiving one-half of an adrenal as a graft showed accelerated hair growth. The animals surviving longer than 30 days were quite inactive and failed to grow as rapidly as litter mates.

AUTOGRAFTS OF AN ENTIRE ADRENAL

In most of the first operations where an entire adrenal was transplanted into the spleen, the animal's life was not maintained longer than 12 days. When the transplants were recovered from these rats, they were found to be degenerate necrotic masses and to contain no

surviving tissue other than remnants of a capsule. The capsule of the gland was left intact at the time of the operation. This probably prevented rapid revascularization and thus complete degeneration followed. When the capsule was punctured or incised and the gland was transplanted, most of the animals survived. Considerable difficulty was experienced, however, in getting such a large piece anchored in the spleen. A better method was then devised which consisted of running a fine thread through the capsule and tying the thread around the spleen. This thread served to anchor the graft in a slit previously made in the spleen and also provided a path for rapid ingrowth of blood vessels. Twelve of 14 animals survived longer than 30 days with such technique. At intervals (44 to 180 days) following the operation the recovered grafts in many instances consisted of very large regenerated masses partly within the spleen and partly on its periphery. Glomerulosa and fasciculata zones were identifiable and the capsule was usually thin. In other instances, the autograft was quite similar in structure to the normal adrenal: all zones were represented and a region filled with blood was present in the center. No medullary material had survived. One rat bore a litter of seven 68 days after the autografting and another rat had a litter of eight 82 days after the transplanting. All the puppies died in one litter and only two of the other litter survived until weaning time. Autografts in the spleen which had maintained life for 40 days were removed from three animals. Death followed on the 6th, 8th and 14th day after removal.

Entire adrenals were difficult to anchor in the liver. A few animals died within 12 days following the transplanting. Grafts when recovered had a thick capsule and a few small islands around the periphery. These islands apparently were not enough to maintain the life of the animal. Most of the animals (12 of 17) which received an entire adrenal survived longer than 30 days. When sacrificed, the autografts were surrounded by a thin capsule and were composed of large compact masses of cells showing no arrangement.

The rats receiving an entire adrenal as an autograft were more active and had better health than those receiving a transplant of a portion of an adrenal. The autografts recovered from animals receiving an entire adrenal as a transplant, while no greater in mass, were better differentiated and the zonation was usually more pronounced than the autograft resulting from a portion of an adrenal.

DISCUSSION

Following bilateral adrenalectomy, adrenal autotransplants in the liver and spleen, although more difficult to anchor, have maintained the lives of hooded and white rats equally as well as autotransplants in the kidney.

When an entire adrenal was tied to the spleen, about 85% of the animals survived longer than 30 days. An entire adrenal was anchored and retained with difficulty in the liver and only 71% of the animals

lived longer than 30 days. Sixty percent survival for more than 30 days resulted after the autografting of one-half of an adrenal in the liver and 69% survival in the case of similar grafts in the kidney. Only 25% survived longer than 30 days with an autograft of one-fourth of an adrenal in either the kidney or the liver. Death often occurred in animals with one-fourth of an adrenal five or six days after the operation while death in animals with an entire adrenal was more common 11 or 12 days after the operation. These experiments showed that the inactivating power of the liver on the cortical hormone was not greater than that of the kidney.

The degenerative and regenerative changes in the adrenal autografts have been followed rather carefully. In general my observations confirm those made by Ingle and Higgins (1938). These authors claim that necrosis begins in the center of the entire organ, extends peripherally, and in 48 hours most of the gland except the capsule is destroyed. In my preparations the moribund cortical cells disappear in a peripheral to central sequence as the polymorphonuclear leucocytes, lymphocytes and macrophages invade them.

Areas of regenerated cortical cells begin to appear beneath the thick capsule within three or four days after the operation and by seven or eight days there are often large islands of cortical tissue. These areas enlarge, usually coalesce, but in many instances remain separated by septa to form an irregular shaped graft. The inequality in the regenerative capacity of the regions of the capsule is difficult to explain as Williams (1947) has pointed out. In the case of a portion of the gland as the autograft, the peripheral capsule immediately surrounds the more centrally located cells which undergo degeneration. As regeneration progresses, islands of newly formed cortical cells appear under the capsule and the capsule usually becomes thinner.

Failure of autografts of one-fourth of an adrenal or one-half of an adrenal to maintain life as well as entire adrenal autografts may have been due to insufficiency of cortical hormone in the implants to carry the animal over the period until the grafts regenerated or to the amount of capsule available for regeneration or to both. Since the gland with the exception of the capsule necroses rapidly after the transplantation, the amount of capsule available for regeneration is probably the determining factor. Hair growth was frequently accelerated in animals receiving one-fourth or one-half of an adrenal indicating cortical insufficiency.

Normal zonation seldom occurred in any of the grafts and for the most part the transplants consisted of cells with a glomerulosa and fasciculata arrangement. The grafts enlarged with time but a reticular zone was as common in 40 day old grafts as it was in much older grafts.

The size reached by the graft has been discussed and investigated at great length by Ingle and Higgins (1938) and Wyman and tum

Suden (1937). The former conclude that the size can . . . "be explained by the assumption that the adrenotropic activity of the pituitary body is responsive to the physiologic requirements for cortin." Several of my rats with autografts had litters yet with apparently normal maternal care they lost most of the litter due to their inability to supply adequate milk. In this instance an adjustment was not made between the physiologic requirements for cortin and the adrenotropic activity of the pituitary body.

SUMMARY

Following bilateral adrenalectomy, either an entire adrenal or a portion of an adrenal was autotransplanted into the liver, the spleen or the kidney of the rat. The amount of the gland transplanted and the percentage of animals surviving for more than 30 days has been as follows: entire in spleen 85%; entire in liver—71%; one-half in kidney—69%; one-half in liver—60%; one-fourth in kidney—25%; and one-fourth in liver—25%.

In surviving transplants, regions of the capsule produced new areas of cells which often remained separated by septa. Normal zonation seldom occurred. Regeneration was equally as good in the liver and spleen as in the kidney.

The tissues of the liver and spleen appear to have no more inactivating effect than the tissues of the kidney on the cortical hormone.

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EFFECTS OF THIOURACIL FEEDING ON RESISTANCE TO LOW ENVIRONMENTAL TEMPERATURE¹

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IN 1943 Leblond and Gross observed that thyroidectomized rats failed to survive at low environmental temperatures unless administered thyroid hormone. Since thiouracil inhibits thyroxin synthesis in the body, impaired survival at low environmental temperatures might also be expected following prolonged administration of this drug. In the present experiment adult female rats were made hypothyroid by thiouracil feeding, and their length of survival determined under cold room conditions with and without thyroxin administration.

PROCEDURE AND RESULTS

Forty-seven female rats of the Sprague-Dawley strain were raised to maturity on a stock ration and selected for the present experiment at approximately 10 weeks of age and an average body weight of 170.1 g. (range 148 to 192 g.). Forty-one rats (group I) were fed a purified ration consisting of thiouracil 0.3%, casein² 22.0%, salt mixture³ 4.5%, whole liver powder⁴ 10.0%, cottonseed oil (Wesson) 10.0% and sucrose 53.2%. To each kg. of the above diet were added the following synthetic vitamins: thiamin hydrochloride 72 mgms., riboflavin 9 mgms., pyridoxine hydrochloride 15 mgms., calcium pantothenate 67.2 mgms., nicotinic acid 60 mgms., 2 methyl-naphthoquinone 5 mgms. and choline chloride 1.2 g. Each rat also received once weekly a vitamin A-D concentrate⁵ containing 150 U.S.P. units of vitamin A and 15 U. S. P. units of vitamin D together with 3 mgms. of alpha-tocopherol acetate. Six rats (group II) were fed a diet identical with the above but with thiouracil omitted and replaced by an

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² Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

³ Salt Mixture No. 1 (Sure, 1941).

⁴ Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

⁵ Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

equal amount of sucrose. Animals were placed in metal cages with raised screen bottoms to prevent access to feces and were fed the above diets *ad lib.* for 4 weeks.

After 28 days of feeding basal metabolic rates were determined for 9 rats selected at random from group I and the 6 animals of group II.⁶ The apparatus used was a closed circuit type with a capacity of 2 liters (Mason and Winzler, 1948). Carbon dioxide was absorbed with sodium hydroxide, and oxygen consumption was determined from pressure changes recorded by means of a water manometer. The respiration chambers were kept at 28°C. and readings obtained were corrected to S.T.P. Food was removed from the animals' cages the evening prior to the metabolism test. At least 6 successive 5-minute intervals were recorded for each animal, with care being taken to record oxygen consumption when animal activity was at a minimum. Following the B.M.R. determinations the above rats were sacrificed and thyroid weights determined. Results are summarized in table 1.

TABLE 1. EFFECTS OF THIOURACIL FEEDING ON OXYGEN CONSUMPTION AND THYROID WEIGHT

Group	Number of animals	Thiou-racil content of ration	Body wt.	Oxygen consumption cc/hr/100 g. body wt. ¹	Change in B.M.R. ²	Thyroid wt. ¹	Thyroid wt. mg./100 g. body wt.
I	9	per cent	g.		per cent	mgms.	
		0.3	174.4	83.3 ± 2.7	-27.9	56.8 ± 6.8	32.3
II	6	0.0	175.6	115.5 ± 6.7	—	10.2 ± 0.5	5.8

¹ Including standard error of the mean.

² Compared to the control B.M.R. of Group II.

Findings indicate that 4 weeks of thiouracil administration resulted in a marked increase in thyroid size and a significant decrease in basal metabolic rate, findings indicative of a hypothyroid state. Surviving rats of group I were then divided into 6 groups and were placed in individual metal cages in a large walk-in refrigerator maintained at a temperature of $2 \pm 1.5^\circ$ C. No change was made in the experimental diet but each rat received daily intraperitoneal injections of the following materials: group A, 1 cc. saline solution; group B, 0.5 cc. adrenal cortex extract;⁷ group C, 2.5 μ g dl-thyroxin;⁸ group D, 5.0 μ g dl-thyroxin; group E, 10.0 μ g dl-thyroxin; and group F, 20.0 μ g

⁶ We are indebted to Miss Betty Tukich and Dr. R. J. Winzler of the Department of Biochemistry and Nutrition, University of Southern California, for the B.M.R. determinations.

⁷ Adrenal Cortex Extract, Wilson Laboratories, Chicago, Ill. The material was administered twice daily in doses of 0.25 cc.

⁸ Thyroxin (Synthetic Cryst.), Roche-Organon, Inc., Nutley, N. J. The material was dissolved in .1 N NaOH, adjusted to a pH of 8.0 and diluted to a volume containing 10 μ g thyroxin per cc.

dl-thyroxin. Injections were begun the day preceding transfer to the cold room. Treatment was continued for 28 days or until death. Food consumption was determined daily for each rat during the first week of treatment. On the 28th day surviving animals were autopsied and thyroid weights determined. Results are summarized in table 2.

TABLE 2. EFFECTS OF dl-THYROXIN ON SURVIVAL TIME AND BODY AND THYROID WEIGHT OF THIOURACIL-FED RATS UNDER COLD ROOM CONDITIONS

Group	Daily treatment received	Number of animals	Initial body weight	Per cent surviving ¹	Average survival time ^{2,3}	Gain in body wt. during expt. ³	Thyroid wt. ³	Thyroid wt. mgms./100 g. body wt.
A	1 cc. saline solution	6	187.8	0.0	5.7 ± 0.6	—	—	—
B	0.5 cc. adrenal cortical extract	6	187.2	0.0	4.3 ± 0.5	—	—	—
C	2.5 cc. dl-thyroxin	5	183.2	100.0	28.0 ± 0.0	13.4 ± 1.9	79.6 ± 5.8	39.4
D	5.0 µg dl-thyroxin	5	188.8	100.0	28.0 ± 0.0	9.2 ± 4.0	43.6 ± 6.8	22.0
E	10.0 µg dl-thyroxin	5	188.6	100.0	28.0 ± 0.0	22.2 ± 4.2	23.0 ± 2.0	10.9
F	20.0 µg dl-thyroxin	5	188.6	100.0	28.0 ± 0.0	19.0 ± 3.0	22.4 ± 1.6	10.7

¹ Experimental period—28 days.

² Averages were computed on the basis of a 28 day survival time for animals alive at the termination of the experiment.

³ Including standard error of the mean.

Findings indicate that thiouracil-fed rats fail to survive under cold room conditions in the absence of thyroid hormone.⁹ Under conditions of the present experiment thiouracil-fed rats in group A survived an average of 5.7 days (range 3 to 7 days). Parenteral administration of adrenal cortical extract was without significant effect on length of survival. Dl-thyroxin, however, at a daily dose as small as 2.5 µg per day was completely effective in maintaining survival during an experimental period of 28 days. All rats administered thyroxin survived and gained weight during the experiment, but differences in growth were not statistically significant. Marked differences in thyroid weight were observed, however, among the 4 groups receiving thyroxin. Thyroid weights were largest for the group administered 2.5 µg thyroxin daily and least for those receiving the 10 or 20 µg levels. At the latter dosage (groups E and F) thyroid weights were significantly less than those observed in similar animals (group I) autopsied prior to the cold room exposure (table 1). The cause of death in groups A and B was not inanition for the food consumption of animals in these groups did not differ significantly in amount from that of animals receiving thyroxin.

⁹ Animals corresponding to these in age and weight and fed a similar diet but with thiouracil omitted survived under cold room conditions without thyroxin administration. The average change in body weight of 6 rats over a 28 day period was -2.2 g. Thyroid weight averaged 11.5 mgrs. (range 10.0-13.8 mgms.) or 6.2 mgms. per 100 g. body weight.

DISCUSSION

Findings indicate that drug therapy may affect resistance to low environmental temperature. In the present experiment thiouracil feeding was shown to impair survival at low environmental temperatures. Unpublished work from this laboratory indicates that similar results can be obtained by feeding promin (sodium p,p'-diaminodiphenylsulfone N,N'-didextrose sulfonate) recently shown by this laboratory to be goitrogenic (Ershoff, 1948). For both drugs thyroxin will significantly prolong survival at low environmental temperatures. Presumably similar results may be obtained with other antithyroid agents such as rape seed, promizole, thiocyanates, sulfonamides and others (Charipper and Gordon, 1947). Failure of survival at low environmental temperatures appears under these conditions to reflect a deficiency of thyroid hormone. The amount of thyroxin necessary to maintain survival, however, appears to be considerably less than the amounts normally secreted under cold room conditions (Dempsey and Astwood, 1943).

SUMMARY

Rats made hypothyroid by thiouracil feeding failed to survive on exposure to a low environmental temperature. Length of survival under these conditions averaged 5.7 days (range 3 to 7 days). One hundred per cent survival over a 28 day period was obtained in animals similarly treated but administered thyroxin.

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COMPARATIVE PARENTERAL THYROXINE-LIKE ACTIVITY OF NATURAL AND SYNTHETIC THYROPROTEINS STUDIED WITH THE GOITER PREVENTION METHOD¹

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LOS ANGELES

INTRODUCTION

MANY investigators have observed that the activity of natural thyroid proteins is greater than an equivalent amount of thyroxine.² The large disagreement in relative oral potencies between thyroid preparations and thyroxine has been explained on the basis of poor absorption of the relatively insoluble thyroxine comparison standard. The smaller but significant differences in parenteral activity have led some workers to attempt to correlate activity with the total iodine content of natural thyroid proteins (Gaddum, 1929-30; Means, Lerman and Salter, 1933; Lerman and Salter, 1934; Salter, 1935). Others have claimed agreement with the thyroxine content when it is assumed that the L-thyroxine contained possesses twice the activity of DL-thyroxine (Palmer and Leland, 1935; Foster et al., 1936, Parkes, 1947). Still others have reported that thyroxine-like activity could not be correlated with either total iodine content or thyroxine content of natural thyroid proteins (Meyer and Wertz, 1939). It has been difficult to settle the problem because of the large experimental errors involved in the biological methods available for the determination of thyroxine-like activity (e. g. basal metabolic rates, guinea pig weight loss, protection against acetonitrile poisoning in mice, amphibian metamorphosis, etc.).

Dempsey and Astwood (1943) were the first to use the ability of thyroxine to depress the thyroid weights of thiouracil-fed rats as a method of determining thyroid secretion rates under various environmental conditions. Mixner, Reineke and Turner (1944) studied the effect of thiouracil and thiourea on the thyroid gland of the chick, and

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² This subject has been reviewed at length in a number of texts including those of Kendall (1929), Harrington (1933), Means (1937), Elmer (1938), Salter (1940).

many of the potential variables in the application of the goiter prevention technique for the biological assay of thyroxine-like activity of thyroidal materials were investigated. These same workers subsequently found that thyroid assays in rats by this technique were directly comparable with results obtained by the standard metabolic method (Reineke, Mixner, and Turner, 1945). Reineke and Turner (1945a) employed this method along with other methods to determine the relative thyroidal potency of L-thyroxine and DL-thyroxine, obtaining essentially the same results with all the different techniques used. This method seemed to be the one of choice and convenience for the biological assay of the thyroxine-like activity of thyroactive substances.

The improvements in the preparation of synthetic thyroproteins by Reineke, Turner and coworkers have been reviewed by Reineke (1946). Reineke et al. (1945) reported agreement between the results of biological assay based on the measurement of basal metabolic rates of guinea pigs, and a modification of the method of Blau (1933, 1935) for the chemical estimation of the thyroxine content of thyroactive iodinated proteins.

This work has been undertaken in order to determine whether the biological activity of natural thyroid corresponds to the thyroxine content of the preparation, and to compare the biological effects of natural and synthetic thyroproteins.

METHODS

Groups of five or six female albino rats of the University of Southern California strain, weighing between 125 and 200 grams, were used throughout these experiments. Prior to the experimental period of 15 days, the animals were fed the stock diet previously employed. The rats were fed ad libitum with stock diet containing 0.3% thiouracil and for 14 days were given daily intraperitoneal or subcutaneous injections of DL-thyroxine or appropriate suspensions or solutions of the natural or synthetic thyroid proteins (the injection volume usually being about 0.20 ml./100 g. body weight, and the thyroxine level in the range of 1 to 10 γ /100 g. body weight). On the fifteenth day the animals were weighed and sacrificed, and the thyroid glands removed and weighed to the nearest 0.1 mg. The room temperature was maintained at $75 \pm 3^\circ$ F., and DL-thyroxine controls were used in each series of experiments. Figure 1 shows a typical DL-thyroxine standard curve obtained in Experiment I, reported in Table 2. From Figure 1, using the method of Dempsey and Astwood (1943), the daily secretion rate of 100 to 200 gram female rats of the University of Southern California strain at 24° C. is seen to be 6.0 γ DL-thyroxine per 100 gram body weight, which is in the same range as the 5.0 γ value reported by Astwood and Bissel (1944) and the 4.1 γ DL-thyroxine reported by Monroe and Turner (1946) under somewhat similar environmental conditions. In each experiment, the biological activity of the thyroactive protein was determined from such a DL-thyroxine-thyroid weight curve run simultaneously with the experimental groups. Tables 2 and 3 include no values which give thyroid weights significantly below that of the normal untreated rat.

MATERIALS

The natural thyroid proteins (D-1, D-2, A, B)³ were analyzed for total iodine by the method of Waters and Beal (1945) and for thyroxine by Blau's (1933) method. Preparations D-1 and D-2 were two samples of USP. desiccated thyroid. A and B were commercially obtained, partially purified thyroid preparations. The synthetic thyroproteins (STP) were prepared essentially by the method of Reineke and Turner (1945b). Particular care

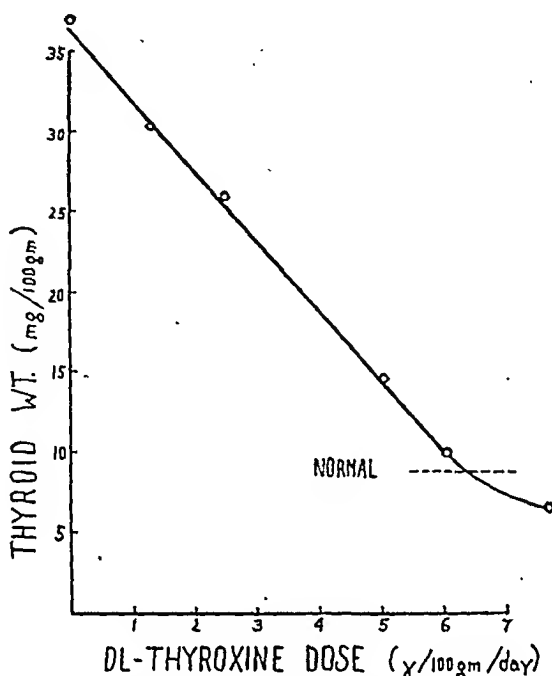


FIG. 1. Typical standard curve showing the effect of DL-thyroxine dose on the thyroid weight in thiouracil-fed rats. Groups of approximately six 150 to 200 gram female rats were fed a 0.3% thiouracil diet for 15 days during which time they received daily intraperitoneal injections of the indicated amount of DL-thyroxine.

was taken to minimize any racemization of L-thyroxine by using sodium bicarbonate instead of sodium hydroxide to dissolve the proteins in all steps of the preparation or testing procedures. STP-1 was subjected to analysis for total iodine and thyroxine. Total iodine was determined again by the method of Waters and Beal (1945). The thyroxine content was determined by the procedure of Reineke et al. (1945) employing a 40% barium hydroxide hydrolysis, and also by Blau's method using a 2 N sodium hydroxide hydrolysis. The average of four individual thyroxine determinations by the former technique was 2.6%. Duplicate analyses on STP-1 by Blau's method gave also 2.6%. Thus, in this limited investigation, there was no evidence of significant differences between Blau's method and that of Reineke et al. for the determination of thyroxine in iodinated proteins. However, the possibility exists that the observed thyroxine values may be high due to the presence of non-thyroxine iodine in the final butyl alcohol fraction. This possibility is strengthened by the biological data reported later. STP-2, 3, 4 were ana-

³ The natural thyroid proteins were provided by the Harrower Laboratory, Inc. Glendale, California.

lyzed for thyroxine by Blau's method in view of the results obtained with STP-1. IC-1 was an iodinated casein product prepared in such a manner that a minimum amount of thyroxine would be formed. Though it contained 4.1% iodine, its thyroxine content was less than 0.1%. The important analytical data on the various proteins employed in this investigation are compiled in Table I.

The proteins were dissolved or suspended in an excess of dilute sodium hydroxide or 1% sodium bicarbonate with the aid of a mortar and pestle, the pH adjusted to 8.0 ± 0.5 and the solution made up to volume. A highly satisfactory suspension of the natural thyroid proteins was obtained in this manner. The synthetic thyroproteins were readily soluble under the above

TABLE 1. TOTAL IODINE AND THYROXINE CONTENTS OF NATURAL AND SYNTHETIC THYROID PRODUCTS

Preparation	Per Cent Total Iodine	Per Cent Thyroxine
Desiccated Thyroid-1	0.52	0.26
Desiccated Thyroid-2	0.64	0.31
Product A	0.99	0.46
Product B	0.27	0.13
STP-1	10.7	2.6
STP-2	7.7	2.4
STP-3	8.6	2.9
STP-4	8.9	2.3
IC-1	4.1	0.1

conditions. DL-thyroxine⁴ was dissolved in a minimum amount of 0.1 N NaOH, the pH adjusted to 8.0 ± 0.5 and the solution made up to volume.

RESULTS

Thyroxine-like activity of parenterally administered natural thyroid proteins. Table 2 lists the data obtained on three independent experiments testing the thyroxine-like activity of the various thyroactive proteins when administered intraperitoneally. In the last column is given the ratio of the biological activity of each preparation to its thyroxine content. If it be assumed that L-thyroxine possesses twice the biological activity of DL-thyroxine, this ratio should have a maximum theoretical value of 2.0. However the ratios for the natural thyroid preparations D-1, A, and B were 4.0, 3.1 and 4.5 respectively. The difference between these values and the theoretical is far greater than can be due to experimental error, and indicates that the biological activity of natural thyroid proteins is some four fold greater than the activity of equivalent doses of DL-thyroxine when both are administered intraperitoneally. In Table 3, a ratio of 5.3 was obtained with desiccated thyroid injected subcutaneously in comparison to

⁴ We are indebted to Dr. Kenneth W. Thompson of Roche-Organon, Inc., Nutley 10, New Jersey for a generous supply of DL-thyroxine.

DL-thyroxine also subcutaneously administered. The potency of subcutaneously and intraperitoneally administered DL-thyroxine has not been found to differ significantly in these experiments.

These data confirm the work of many earlier investigators who were unable to account for all the thyroxine-like activity of natural

TABLE 2. THE THYROXINE-LIKE ACTIVITY OF NATURAL AND SYNTHETIC THYROACTIVE PROTEINS WHEN ADMINISTERED BY INTRAPERITONEAL INJECTION

0.3% Thiouracil plus	No. of Animals	Administered Dose		Average Thyroid Weight	Biological Response ¹	Biological Response	
		(Mg.)	(γ Thyrox.)	(gm.)	(Mg./100 gm.)	(γ Thyrox.)	Adm. dose
		(100 gm.)	(100 gm.)	(gm.)	(Mg./100 gm.)	(γ Thyrox.)	Adm. dose
<i>Experiment 1</i>							
Controls	8	—	—	166	36.8 \pm 7.9 ¹	—	—
DL-thyroxine	6	—	5.00	171	14.2 \pm 3.1	5.00	1.00
Desiccated Thyroid-1	5	0.509	1.56 ²	159	9.5 \pm 1.8	6.00	3.90
Prod. "B"	5	1.00	1.31	162	9.7 \pm 2.2	5.90	4.50
Prod. "A"	5	0.374	1.72	161	12.4 \pm 2.8	5.75	3.30
STP-1	6	0.080	1.56	165	28.1 \pm 4.8	1.70	1.10
L-Diiodotyrosine	4	25.0	—	150	10.8 \pm 1.3	5.70	—
<i>Experiment 2</i>							
Controls	6	—	—	147	33.0 \pm 2.6	—	—
DL-thyroxine	6	—	6.00	176	9.3 \pm 1.8	6.00	1.00
Desiccated Thyroid-1	6	0.539	1.40	192	10.4 \pm 1.9	5.60	4.10
Prod. "B"	6	0.840	1.10	184	14.8 \pm 1.3	5.00	4.50
Prod. "A"	6	0.391	1.80	192	10.5 \pm 1.4	6.20	3.20
STP-1	6	0.193	5.00	193	11.6 \pm 1.7	5.60	1.10
L-Diiodotyrosine	6	15.0	—	153	17.8 \pm 3.2	4.20	—
<i>Experiment 3</i>							
Controls	8	—	—	192	27.5 \pm 4.3	—	—
DL-thyroxine	5	—	4.00	182	10.6 \pm 1.8	4.00	1.00
Desiccated Thyroid-1	5	0.431	1.12	168	8.6 \pm 0.4	4.40	3.90
Desiccated Thyroid-2	5	0.362	1.12	182	10.3 \pm 1.1	4.00	3.60
Prod. "B"	5	0.763	1.00	169	8.7 \pm 2.5	4.35	4.40
Prod. "A"	6	0.307	1.41	178	10.3 \pm 2.9	4.10	2.90
STP-1	5	0.164	4.10	167	8.9 \pm 1.7	4.30	1.00
STP-2	7	0.146	3.50	169	14.0 \pm 4.2	3.15	0.90
STP-3	4	0.121	3.50	188	14.5 \pm 3.0	3.00	0.85
STP-4	6	0.152	3.50	166	13.2 \pm 3.4	3.35	0.95
IC-1	5	2.60	—	171	29.0 \pm 3.4	0.00	0.00
<i>Normal Controls</i>							
No Thiouracil	9	—	—	230	9.5 \pm 1.3	—	—

¹ Average deviation.

² Based on chemical analysis.

³ Estimated to the nearest 0.05 γ from DL-thyroxine dose (I.P.)-response curve for particular experiment.

thyroid proteins in terms of their thyroxine content. Foster and co-workers (1936), however, have reported that the activity of natural thyroid proteins is accounted for if it be assumed that D-thyroxine is inactive and that all the thyroxine present in the thyroid proteins is in the L-form. However, these findings were based on a comparison of orally administered thyroid protein with injected thyroxine. Preliminary data obtained by us indicate that orally administered natural thyroid preparations are appreciably less potent than when they are given parenterally, making it clear that comparison of activities should only be made when both materials are given by the same route. When this is done, a striking excess activity is observed for natural thyroid proteins.

Thyroxine-like activity of parenterally administered synthetic thyroid proteins. In contrast to the results obtained for natural thyroid proteins, synthetic thyroproteins showed less thyroxine-like activity than expected from their L-thyroxine content, the ratio of biological activity to chemical thyroxine content averaging 1.0. It is not believed that the preparation of iodinated proteins involves any extensive racemization of the L-thyroxine formed. Indeed, Reineke and Turner (1943) have isolated L-thyroxine from similarly prepared materials. Thus, the data presented in Tables 2 and 3 leads to the conclusion that the thyroxine-like activity of parenterally administered synthetic thyroproteins is less than that predicted from their L-thyroxine content. This finding conflicts somewhat with the ex-

TABLE 3. THE THYROXINE-LIKE ACTIVITY OF NATURAL AND SYNTHETIC THYROID PROTEINS WHEN ADMINISTERED BY SUBCUTANEOUS INJECTION

0.3% Thiouracil plus	No. of Animals	Administered Dose		Av. Wt. Animals (gm.)	Average Thyroid Weight (Mg./100 gm.)	Biological Response ¹ (γ Thyrox.)	Biological Response Adm. dose
		(Mg.)	(γ Thyrox.)				
		(100 gm.)	(100 gm.)				
Thiouracil Controls	6	—	—	130	30.0 \pm 2.9 ¹	—	—
DL-thyroxine	6	—	2.00	132	24.4 \pm 2.4 ¹	2.00	1.0
DL-thyroxine	6	—	4.00	127	13.0 \pm 2.2	4.00	1.0
STP-1	6	0.135	3.50 ²	134	21.3 \pm 2.4	2.20	0.65
Desiccated Thyroid-1	4	0.336	0.675 ²	133	11.0 \pm 0.3	4.60	5.30

¹ Average deviation.

² Based on chemical analysis for thyroxine.

³ Estimated to the nearest 0.05 γ from DL-thyroxine dose (subcutaneous)-response curve for particular experiment.

tensive data of Reineke et al. (1945) who reported that in a group of 15 thyroactive iodinated casein preparations the chemical thyroxine content was only about 10 % greater than the biological assay of L-thyroxine as determined from their influence on the metabolic rates of guinea pigs. This would correspond to a ratio of 1.8 as defined above. The possibility that our chemically determined thyroxine values are higher than those actually existing in the iodinated casein has not been sufficiently excluded, however, and this may account for our ratios of less than 2.0. Preliminary tests have indicated a greatly diminished oral activity of thyroactive iodinated casein in the rat, the thyroxine-like activity being approximately 15% of the same dose administered intraperitoneally. This is in qualitative agreement with the observations of Turner and Reineke (1946) on the utilization of synthetic thyroproteins by sheep.

DISCUSSION

There are several possible explanations which might account for the excess parenteral potency of natural thyroproteins as compared to crystalline thyroxine.

Differences in rates of absorption, excretion, and destruction of

thyroxine in natural and synthetic thyroid proteins and in the DL-thyroxine standard may be the explanation of these observations. It is not unlikely that the rate of utilization of proteins and free amino acids when given by this route may vary considerably, and differences in excretory rates are equally possible. The order of solubility of thyroactive proteins at $\text{pH } 8.0 \pm 0.5$ was $\text{STP} > \text{A} > \text{D} \cong \text{B}$; while the order of activity was $\text{B} > \text{D} > \text{A} > \text{STP}$. This might be interpreted as relatively slower, more effective parenteral release of the less soluble natural thyroactive proteins.

The possibility of a rather specific synergism between thyroxine and the diiodotyrosine-like fractions of natural thyroid proteins has been postulated by many investigators. Studies of thyroxine-like activity of 3,5-diiodo-L-tyrosine indicate that while this substance is active in the goiter prevention test, it is less than 0.1% as active as DL-thyroxine as shown in table 2. Thus, the greater activity of natural thyroid proteins cannot be accounted for directly by their diiodotyrosine content. There was no evidence of any thyroxine-like activity of diiodotyrosine in IC-1 when given at a level of 0.21 mg. diiodotyrosine equivalent/100 grams of body weight. The diiodotyrosine-thyroxine ratio was as great as 5:1 in the STP preparations with no indication of extra-thyroxine potency. Thus, only a very special synergism, perhaps dependent upon ease of *in vivo* conversion of diiodotyrosine to thyroxine or a thyroxine-diiodotyrosine peptide, could explain the observed greater activity of natural thyroid proteins on the basis of the non-thyroxine iodine fractions.

Harington and Randall (1929) have been able to account for the organic iodine of the thyroid in the forms of 3,5-diiodo-L-tyrosine and L-thyroxine, suggesting that other iodine containing compounds with thyroxine-like activity do not exist in the thyroid gland. However, no decisive information can yet be marshalled to exclude or accept the suggestion that peptides containing both thyroxine and diiodotyrosine may be more active than thyroxine alone. An argument against this possibility is the recent observation of Taurog and Chai-koff (1948) in which free thyroxine has been demonstrated in rat plasma.

The possibility also exists that hormones unrelated to thyroxine which affect metabolism or the pituitary-thyroid axis may occur in the thyroid gland (Mansfeld, 1943), and thus result in the excess potency observed in this work.

SUMMARY

The goiter prevention method has been employed to determine the thyroxine-like activity of parenterally administered natural and synthetic thyroid proteins. Natural thyroid proteins have consistently shown more thyroxine-like activity than could result from their thyroxine content, even assuming that L-thyroxine possesses twice

the activity of DL-thyroxine. However, when compared on the same basis, synthetic thyroproteins have revealed only one-half the parenteral activity expected from their L-thyroxine content as determined by the available chemical methods.

Suggested explanations of these effects include possible differences in rates of absorption, utilization and excretion of the thyroactive proteins and the DL-thyroxine comparison standard, a possible specific synergism of diiodotyrosine-like and thyroxine-like fractions of natural thyroid proteins, and the possible presence of thyroid hormones unrelated to thyroxine which affect the pituitary-thyroid axis.

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A MODIFIED TURBIDIMETRIC METHOD FOR THE ASSAY OF HYALURONIDASE

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WITH the possible exception of the turbidimetric assay, no reliable or practical method exists for the accurate determination of the potency of hyaluronidase preparations. Although the turbidimetric method is by no means ideal, it does lend itself to the performance of a large series of tests and it is accurate to within 10 per cent (Meyer, 1947).

A modification of the turbidimetric hyaluronidase assay is being used in this laboratory with good results. It is based on the turbidimetric method as developed by Seastone (1939, 1943), Kass and Seastone (1944), and Leonard, Perlman and Kurzrok (1946). The modification in assay procedure is the result of the daily handling for approximately eleven months of 10 to 20 assays under practical working conditions. With careful attention to details, the method is accurate to within ± 5 per cent.

MATERIALS AND METHODS

Standard hyaluronidase preparations. In order to minimize day-to-day deviation of assay values, a dry hyaluronidase preparation (bull testis) is set aside as the standard enzyme. The preparation is run side by side with the unknown test solutions. For convenience, the standard is assigned an arbitrary potency of *1.0 turbidity reducing unit per milligram*. A standard curve is obtained by plotting the average of two turbidity readings against five enzyme concentrations (.05, .04, .03, .02, .01 mg/ml). A second standard, the potency of which was established by repeated tests, is also assayed daily with the unknowns. It is, therefore, possible to determine the accuracy of the results on any one day. The potencies of the enzyme samples are computed by comparison with the standard curve. The turbidity readings are then converted to milligrams as compared with the standard (see table 1).

Estimation of hyaluronidase activity. For the determination of enzyme activity, a dry hyaluronidase sample, previously diluted to 1 mg/ml with distilled water, is maintained at 24°C. for 30 minutes. The hyaluronidase is further diluted with 0.02 M acetate buffer pH 6.0 to the appropriate dilution range. The varying concentrations of enzyme, in duplicate, are now distributed in a series of tubes and the volume is adjusted to 0.5 ml with buffer solution. To each tube is added 0.5 ml of a purified potassium hyaluronate (umbilical cord) dissolved in 0.1 M acetate buffer pH 6.0 to a concentration

HYALURONIDASE ASSAY

TABLE 1. ILLUSTRATING METHOD USED FOR THE CALCULATION OF HYALURONIDASE ACTIVITY (See Fig. 1)

Enzyme concentrations (mg./ml.)	.05	.04	.03	.02	.01
Turbidity readings (unknown)	2.5	10	21	32.5	46.5
Standard curve conversion (mg.)	—	.05	.0384	.0264	.0134
Corrected enzyme conc. per milligram	—	1.25	1.28	1.32	1.33
Results: 129.5 TRU/mg.					

of 0.2 mg/ml. The tubes are thoroughly mixed, incubated in a 37°C. water bath for 30 minutes and immersed in an ice bath for 5 minutes. To each tube 3 cc of 0.5 M acetate buffer pH 4.2 and 1 cc of 1:5 dilution of horse serum pH 4.2 are added and mixed. The turbidities are allowed to develop for 30 minutes in a 24°C. incubator and read immediately in a Klett-Sumerson Photoelectric Colorimeter with a red filter No. 66.

RESULTS

Stability of hyaluronate-hyaluronidase-serum mixture. The instability of the enzyme-substrate-serum precipitate during the time

TABLE 2. STABILITY STUDY OF HYALURONATE-HYALURONIDASE SERUM MIXTURE

Hyaluronidase sample	Turbidity reducing units per milligram					Per cent change in activity			
	0	Exposure time (minutes)							
		10	60	90	150	10	60	90	150
5 C									
A	1.00	1.17	1.34	1.54					
B	2.04	2.31	2.75	2.91	1.62	17	34	54	62
C	6.89	6.77	8.18	8.30	9.37	12	34	42	45
24 C.						-2	18	20	36
A	1.00	1.05	1.25	1.49	1.54				
B	1.96	2.00	2.44	2.49	2.90	5	25	49	54
C	6.51	6.98	8.34	8.87	9.06	2	24	27	48
						6	28	36	39

which elapses between sampling and reading the turbidity was reported by Kass and Seastone (1944). These authors found a 15 per cent decrease in turbidity after 2 hours standing at a room temperature. The observations have been confirmed in our laboratory. It was found that the changes occur most frequently in systems containing highly active hyaluronidase preparations. Table 2 summarizes the stability of three hyaluronidase preparations of different purity which were incubated with the standard amount of potassium hyaluronate-acidified serum for the usual 30 minute period (24°C.) directly incubated at 24°C. and 5°C. and assayed at intervals over a period of 2½ hours. The results indicate a marked decrease in turbidity, i.e., increase in activity, of partially purified hyaluronidase A that ranges from 5 to 62 per cent. The more purified preparations B and C show changes between 2 and 48 per cent. It is noted that the intensity of turbidity change decreases at 5°C. with purity of enzyme.

Since the time consumed in reading the turbidities of 15 duplicate samples is approximately 75 minutes, the performance of a large number of tests on any one day was jeopardized by the misleading assay values. This discrepancy was eliminated by the adoption of a "stagger" plan, i.e., two samples in duplicate were diluted, mixed with the standard amount of polysaccharide and incubated at 37°C.

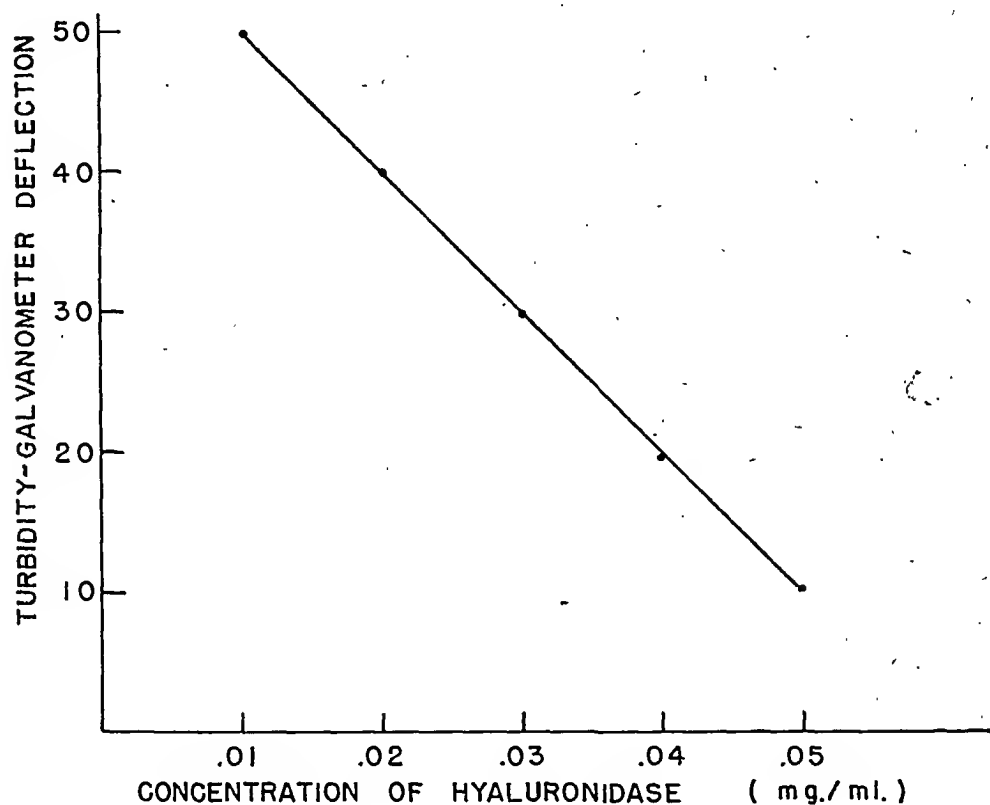


FIG. 1. A Typical Standard Curve

in a water bath; a 10 minute interval was permitted to elapse before the remaining samples, in groups of two, were similarly set up. This modification standardizes the time factor between sampling and reading the turbidity in a large series of assays. The results on any given preparation were found to be highly reproducible.

DISCUSSION

The turbidimetric method for the assay of hyaluronidase defines a *turbidity reducing unit* (TRU) as that amount of enzyme which reduces the turbidity given by 0.2 mg of hyaluronic acid to that given by 0.1 mg (Kass and Seastone, 1944; Leonard Perlman and Kurzrok, 1946; Meyer, 1947). However, samples of purified hyaluronic acid fractionated in this laboratory and in other laboratories were found to possess variable reaction rates with the same concen-

trations of standard enzyme under similar experimental conditions. It is therefore obvious that a hyaluronidase or hyaluronic acid standard is essential to maintain a TRU of comparable value.

In the daily performance of a large series of assays, an adequate supply of hyaluronic acid for an extended period of time was not available. This necessitated the utilization of several batches of the substrate with varied depolymerization properties. In order to minimize these uncontrollable fluctuations and at the same time establish a unit of comparative value, one partially purified hyaluronidase preparation was assigned an arbitrary potency of 1.0 TRU/mg and retained as the standard. The enzyme activity of the unknowns may then be expressed in TRU/mg equivalent of standard irrespective of the quality of the substrate. A second standard not only served as a check on the reacting substances in the test but also indicated the accuracy of the method by establishing on the basis of 150 assays an experimental error of ± 5 per cent.

SUMMARY

A turbidimetric assay for determining the hyaluronidase activity of tissue extracts is described. The method simplifies involved procedures, is accurate to within 5 per cent and appears to be of value in the performance of a large number of assays. The activity of unknowns is determined by comparison with a standard hyaluronidase preparation.

The adoption of a "stagger" plan eliminated discrepancies in assay values which resulted whenever a substantial time interval elapsed between sampling and reading turbidities.

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ACKNOWLEDGMENT

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THE EFFECTS OF PITUITARY AND NON-PITUITARY GLAND FACTORS ON THE FORMATION OF INTRACELLULAR COLLOID DROPLETS IN THE THYROID EPITHELIUM OF HYPOPHYSECTOMIZED RATS¹

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THE PRESENCE of droplets of colloid within the thyroid epithelium was first described in 1872 by Verson in fresh tissue. They were soon after described in fixed thyroid tissue by Gutknecht (1885) and Biondi (1888, 1892). Early workers described their occurrence following the injection of various chemicals (Andersson, 1894; Hurthle, 1894; Galeoti, 1897), or after certain operative procedures unrelated to the thyroid or pituitary glands (Hurthle, 1894; Thomas, 1934). More recent work established a relationship between the formation of intracellular colloid droplets and thyrotrophic hormone. Droplets form promptly after the administration of exogenous thyrotrophic hormone to intact animals (Grant, 1930, 1931; Severinghaus, 1933; Uhlenhuth, 1936; Okkels, 1934; Ponse, 1938; Aleschin, 1935, 1939; Ponse and Altschuler, 1940; De Robertis, 1941, 1942) with dosages of hormone smaller than those needed to increase thyroid cell height of gland weight (De Robertis and Del Conte, 1944; Dvoskin, 1947b). They appear also after presumably enhanced endogenous thyrotrophic hormone production caused by partial thyroidectomy (Hurthle, 1894; Tschassovnikoff, 1915; Ishimaru, 1926; Satwornitzkaja and Simnitsky, 1932) and by thiourea or thiouracil administration (Thomas, 1944; Grasso, 1946; Dvoskin, 1947c.)

However, droplet formation may not always be a specific response to thyrotrophic hormone. Thus Langendorff (1889), Lubeke (1902), Champy (1915), Demuth (1932) and, more recently, Dvoskin (1947d) demonstrated that, in vitro, colloid infiltrates the epithelium and forms intracellular colloid droplets in the absence of added thyrotrophic hormone. This does not deny the fact that droplet formation may also be induced in vitro under certain conditions with thyrotrophic hormone (Junqueira, 1947; Dvoskin, 1947d), but suggests that this response lacks specificity for thyrotrophic hormone.

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² Ayerst, McKenna and Harrison Fellow of the American Association for the Study of Internal Secretions, 1947-1948.

The following experiments were undertaken in order to study the factors related to droplet formation in the thyroid of living animals.

METHODS

The first group of experiments was performed on hypophysectomized rats in order to rule out the possibility of participation of the test animals' own pituitary gland in the reaction. Male rats of the Long-Evans strain, ranging from 50 to 80 days of age, were hypophysectomized by the parapharyngeal approach. Completeness of removal of the hypophysis was ascertained in all cases by study of serial sections of the pituitary capsules. The solutions tested were administered by intracardiac injection one day after operation. One series of experiments was designed to determine the influence of time after injection on the response due to a fixed dosage of pituitary extract. Since the maximum number of droplets formed one hour after injection, another series of experiments was performed to study the response due to various dosages of this pituitary extract³ and of a standard pituitary gland preparation⁴ at one hour after injection. Since thyroxine was later to be used to inhibit the secretion of thyrotrophic hormone in intact animals, it was important to determine whether it or iodide could directly inhibit droplet formation in the thyroid of hypophysectomized rats due to simultaneously administered thyrotrophic hormone.

The specificity of droplet formation by thyrotrophic hormone was tested by administering toxic dosages of typhoid vaccine or histamine diphosphate or pilocarpine hydrochloride to hypophysectomized rats, and studying the effect on droplet formation one hour after the first injection. Typhoid vaccine and the lower dosages of histamine and pilocarpine were administered by a single intra-cardiac injection. The higher dosages of histamine and pilocarpine were administered in divided dosages over an hour period. Half the dosage was administered by intracardiac injection. The remainder was given subsequently by intraperitoneal injection.

Another series of experiments was performed to determine the number of droplets which formed at 1, 8 or 20 days after hypophysectomy. These animals were given an intracardiac injection of 50 μ g. of hog pituitary extract or 1 cc. of typhoid vaccine and autopsied one hour later.

Fresh unmodified pooled sera from rats subjected to the following experimental conditions were injected into the heart of hypophysectomized rats in 2 cc. amounts in order to determine the effect on droplet formation: a. Sera from normal young female rats housed at room temperature. b. Sera from young female rats exposed to 5° C. for 16 hours prior to exsanguination. Exposure to cold (Cramer, 1928; Kenyon, 1933; Kuschinsky, 1935; Woitke-witch, 1935; Baillif, 1937; Uotila, 1939; Starr and Roskelley, 1940) is known to result in stimulation of endogenous thyrotrophic hormone secretion as evidenced by increase in thyroid cell height. c. Some of this serum (b) was

³ The pituitary extract used was a five-year-old flavianic acid precipitate prepared from hog whole pituitary glands by Dr. George Smelser. This extract contained large amounts of thyrotrophic hormone, and was the same as that assayed on chicks in an earlier paper (Dvoskin, 1947b). A generous supply of this extract was available through the kindness of Dr. Smelser.

⁴ Held for the Health Organization of the League of Nations by the National Institute for Medical Research, London, and obtained from the Committee of Revision of the Pharmacopocia of the United States of America, Philadelphia, Pa.

also incubated at room temperature with thiouracil,⁵ in amounts of 1 mg. per cc. of serum, for 30 minutes prior to injection. This was done to determine whether thiouracil could reactivate (Rawson, Albert, McArthur, Merrill, Lennon and Riddell, 1946) or augment (Albert, Rawson, Merrill, Lennon and Riddell, 1947) any thyrotrophic hormone which might be present in the serum. d. Serum from similarly cold exposed rats which had been injected twice subcutaneously with 100 μ g. of sodium iodide in 0.2 cc. of water, once at the start and once halfway through the period of cold exposure. This experiment was performed since Rawson, Moore, Peacock, Means, Cope and Riddell (1945) showed that iodide prevented the inactivation of thyrotrophic hormone by thyroid tissue in vitro. e. Sera from thyroidectomized young female rats kept at room temperature for 17 hours or 10 days following thyroidectomy and prior to exsanguination. f. Sera from rats exposed to 5° C. for 17 hours following thyroidectomy, and prior to exsanguination. g. Sera from normal young male rats fed 0.1% thiouracil in the diet for 7 days prior to exsanguination. The feeding of goiterogenic drugs (MacKenzie and MacKenzie, 1943; Astwood, Sullivan, Bissell and Tyslowitz, 1943) is known to stimulate secretion of thyrotrophic hormone as evidenced by increase in thyroid cell height and gland weight.

Human sera, obtained from normal patients and from patients suffering from hyperthyroidism were also injected. The sera from the latter were taken before or after therapy with iodine or thiouracil and also from post-thyroidectomy cases. Since the post-operative human sera were always toxic or lethal to rats, control sera from patients following other operations were tested to determine whether they also stimulated droplet formation. Some of the hypophysectomized rats were injected with 1.0 cc. of Tyrode's solution, and served as injected controls.

In the experiments with normal rats, droplet formation was induced by exposure to cold and by thiouracil feeding. The role of the pituitary gland in the maintenance of the droplets formed by these procedures was studied by hypophysectomizing these animals and then continuing cold-exposure or thiouracil-feeding.

Other experiments were designed to determine whether droplet formation could be induced in the thyroid of the hypophysectomized rat by cold-exposure or thiouracil injections. The increase in thyroid cell height or gland weight does not occur in hypophysectomized animals exposed to cold (Wolf and Greep, 1937; Uotila, 1939) or fed goiterogenic drugs (Astwood, et al., 1943).

The role of the pituitary gland was tested indirectly by studying the effects of thyroxine administration on droplet formation due to concurrent exposure to cold or thiouracil administration in normal rats. Thyroxine administration prevents the increase in thyroid cell height and gland weight in normal rats fed goiterogenic drugs (MacKenzie and MacKenzie, 1943; Astwood, et al., 1943; Reineke, Mixner and Turner, 1945), presumably by suppressing thyrotrophic hormone secretion. Thyroxine was administered in two forms. One group was treated with subcutaneous or intrasplenic implants of 1.4 mg. to 1.8 mg. pellets of mono-sodium d,1-thyroxine.⁶ Lower

⁵ The thiouracil was supplied through the courtesy of the Calco Chemical Division of the American Cyanamid Company.

⁶ The mono-sodium thyroxine was supplied through the courtesy of Hoffmann-LaRoche.

dosages were administered by implanting similar pellets made using cholesterol as a diluent. Since the weight changes in these pellets were very small, the dosages which they provided could only be roughly estimated. In order to study the effect of a known dosage another group received daily subcutaneous injections of 7.5 μ g. d,l-thyroxine in aqueous solution. Animals injected daily with 8.9 mg. NaI served as controls for the thyroxine-treated animals.

Since the adrenal cortex (Long, 1947; Sayers and Sayers, 1947) and medulla (Cannon, Querido, Britton, and Bright, 1927; Cramer, 1928) are known to be stimulated to secrete by exposure to cold, it was of interest to determine whether droplet formation in the thyroid was related to the activation of this gland. In these experiments, the response of adrenalectomized rats to cold-exposure was studied. In others, the effect of injections of either epinephrine or adrenal cortical extract on droplet formation in normal rats kept at room temperature was determined.

At the end of each experiment, the animals were killed by illuminating gas, the thyroids were immediately dissected free under a binocular microscope, and weighed to the nearest milligram. The glands were then promptly fixed in Carnoy's fluid,⁷ imbedded in paraffin, and sectioned at 4 μ . Sections were stained by the modified Heidenhain-Azan stain.

The number of intracellular colloid droplets in the thyroid epithelium of each animal was determined by totalling the number of droplets in the epithelium of 25 cross sections of follicles in a section through the mid-portion of the gland. A 10 \times ocular and a 90 \times oil immersion objective lens were used. In order to determine if there was any response in cell heights under conditions which were sufficient to stimulate droplet formation, cell heights were measured by a method described earlier (Dvoskin, 1947a, 1947b, 1947c). The mean and standard deviation of the number of colloid droplets and of the cell heights and weights of the thyroids of each group, and P values comparing the means of different groups, were calculated

RESULTS.

1. *Effect of Pituitary Extracts in Hypophysectomized Rats.* The thyroid epithelium of adult male hypophysectomized rats contained no colloid droplets one day after operation (Fig. 1). A single intracardiac injection of 50 μ g. of hog pituitary extract produced large numbers of droplets as early as 30 minutes after injection. The number of droplets was maximal at 60 minutes after injection and only a few droplets could be found after 180 minutes (Table 1).

An injection of 1 μ g. of hog pituitary extract did not stimulate droplet formation within one hour after its injection. However, as little as 5 μ g. of the extract stimulated the formation of a small number of intracellular colloid droplets (Table 2). Increasing dosages, up to 100 μ g., stimulated formation of increasing numbers of droplets (Fig. 2). Statistically significant differences were found between the means of the groups with but slight overlap between groups (Table 2). The difference in cell height between animals treated with

⁷ This fixative preserves the intracellular colloid droplets in rat thyroid epithelium.

100 μ g. of the hog pituitary extract and that of untreated controls was at the borderline of statistical significance, while the cell height of animals treated with 50 μ g. was not significantly increased over the controls.

The International Preparation of the Anterior Pituitary Gland Substance of the Ox was tested for its effect on droplet formation at two dosage levels. The slope of the line which may be plotted from the number of droplets which appeared following injection of this preparation closely resembles that obtained with the hog pituitary

TABLE 1. INTRACELLULAR COLLOID DROPLETS IN THYROID EPITHELIUM OF MALE RATS ONE DAY AFTER HYPOPHYSECTOMY AND AFTER VARYING TIMES AFTER INTRACARDIAC INJECTION WITH 50 μ g. OF HOG PITUITARY EXTRACT IN 0.5 CC. OF WATER

Time—min. after injection	No. of ani- mals	No. of droplets in 25 follicles Average $\pm \sigma$	Actual results	P ¹
30	5	387 \pm 67	314, 319, 410, 436, 458	
60	5	432 \pm 65	352, 393, 397, 509, 509	>0.2
120	5	114 \pm 26	0, 10, 12, 26, 66	<0.01
180	5	15 \pm 4	0, 0, 2, 3, 10	<0.01

¹ P value of group compared with one treated for 30 minutes.

extract. Comparison of the activity of a solution prepared from 150 μ g. of the International Preparation with the dosage-response curve of the hog pituitary preparation shows that it was roughly as active as 3.5 μ g. of the hog pituitary extract. The solution prepared from 750 μ g. of the International Preparation was roughly as active as 18 μ g. of hog pituitary extract. Albert, Rawson, Merrill, Lennon and Riddell (1946) reported that 15 mg. (15,000 μ g.) of the International Preparation was equal to one Junkmann-Schoeller Unit. On this basis one may say that this test method is able to detect the activity in a solution prepared from 150 μ g. (Table 2) or 0.01 Junkmann-Schoeller Unit.

The administration of 10 μ g. of d,l-thyroxine, a dosage which will inhibit droplet formation in normal animals, did not influence droplet formation due to the simultaneous injection of 50 μ g. of pituitary extract (Table 2). The injection of 100 μ g. of NaI also did not influence the results of injection of pituitary extract (Table 2).

2. *Specificity of the Reaction.* The injection of 1 cc. of typhoid vaccine invariably resulted in the formation of colloid droplets in the thyroids of hypophysectomized rats (Table 2, Fig. 4). The injection of 1 mg. of histamine stimulated droplet formation in half of the animals treated with this dosage, while 3 mg. of histamine stimulated droplet formation in all animals (Table 2, Fig. 3). Similarly, 1 mg. of pilocarpine elicited droplet formation in one of six animals, while 15 mg. resulted in droplet formation in all animals (Table 2).

TABLE 2. INTRACELLULAR COLLOID DROPLETS OF THYROID EPITHELIUM OF ADULT MALE RATS, ONE DAY AFTER HYPOPHYSECTOMY AND ONE HOUR AFTER THE INTRACARDIAC INJECTION OF THE FOLLOWING MATERIALS

Group	No. of animals	No. of intracellular colloid droplets in 25 follicles Average $\pm \sigma$	Actual results	P ¹
Uninjected Controls	10	0 \pm 0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0.	
1 cc. Tyrode's Solution	3	0 \pm 0	0, 0, 0.	
1 μ g. Hog. Pit. Extr.	5	0 \pm 0	0, 0, 0, 0, 0.	
5 μ g. Hog. Pit. Extr.	5	25 \pm 17	7, 8, 28, 41, 42.	<0.01
10 μ g. Hog. Pit. Extr.	5	51 \pm 12	38, 40, 48, 63, 65.	<0.01
25 μ g. Hog. Pit. Extr.	5	291 \pm 69	235, 242, 246, 360, 373.	<0.01
50 μ g. Hog. Pit. Extr.	5	432 \pm 65	352, 393, 397, 509, 509.	<0.02
100 μ g. Hog. Pit. Extr.	5	647 \pm 125	510, 526, 670, 692, 839.	<0.02
50 μ g. Hog. Pit. Extr. and 10 μ g. thyroxine	5	414 \pm 66	326, 378, 424, 442, 500.	>0.07
50 μ g. Hog. Pit. Extr. and 100 μ g. NaI	5	414 \pm 37	371, 385, 420, 430, 465.	>0.3
150 μ g. Ox. Pit. (Int. Standard)	5	13 \pm 6	6, 9, 11, 19, 20.	
750 μ g. Ox. Pit. (Int. Standard)	5	174 \pm 52	112, 143, 181, 183, 251.	
1 cc. Typhoid Vaccine	6	46 \pm 24	17, 21, 44, 55, 56, 82.	
1 mg. Histamine Diphosphate	8	41 \pm 67	0, 0, 0, 0, 4, 28, 139, 158.	
3 mg. Histamine Diphosphate	6	46 \pm 27	15, 25, 35, 47, 71, 84.	
1 mg. Pilocarpine Hydrochloride	6	5 \pm 67	0, 0, 0, 0, 0, 30.	
15 mg. Pilocarpine Hydrochloride	6	33 \pm 23	7, 18, 24, 26, 53, 67.	
2 mg. Thiouracil	5	0 \pm 0	0, 0, 0, 0, 0.	

¹ P value comparing group with one treated with the next lower dosage. Groups treated with thyroxine or NaI and hog pituitary extract are compared with the one treated with the same amount of pituitary extract alone.

There was a rough correlation between the toxic effect of materials administered and the number of droplets which formed. In general, the larger and more toxic dosages were more effective in stimulating droplet formation than the lower less toxic dosages. In most cases, the injection of a material which prostrated the animals stimulated droplet formation. The droplets which formed after injection of these toxins were most numerous in the peripheral follicles, and were

never as numerous as after injection of the large dosages of non-toxic pituitary extract.

3. *Effect of Time After Hypophysectomy on Droplet Formation.* The number of droplets which formed one hour after the intracardiac injection of 50 μ g. of hog pituitary extract was markedly lower at 8 than at 1 day after hypophysectomy. A further reduction in the

TABLE 3. INTRACELLULAR COLLOID DROPLETS AND CELL HEIGHT OF THYROID EPITHELIUM OF HYPOPHYSECTOMIZED MALE RATS AT VARIOUS TIMES AFTER OPERATION AND ONE HOUR AFTER INJECTION OF PITUITARY EXTRACT OR TYPHOID VACCINE

Treatment	Days after hypophysectomy	No. of animals	No. of droplets in 25 follicles		Cell height—microns Av. $\pm \sigma$
			Av. $\pm \sigma$	Counts in individual animals	
50 μ g. Hog. Pituitary Extract	1	5	432 \pm 65	352, 393, 397, 509, 509.	7.8 \pm 0.9
	8	3	222 \pm 39	196, 204, 268.	4.9 \pm 0.2
	20	3	70 \pm 22	49, 70, 92.	3.9 \pm 0.3
1.0 cc. Typhoid Vaccine	1	6	46 \pm 24	17, 21, 44, 55, 56, 82.	7.1 \pm 0.5
	8	4	8 \pm 5	3, 3, 12, 12.	5.0 \pm 0.2
	20	4	0 \pm 0	0, 0, 0, 0.	3.4 \pm 0.3

number of droplets occurred if a 20 day period elapsed before the injection (Table 3).

A progressive decrease in the number of droplets was also observed following the injection of 1 cc. of typhoid vaccine at increasing periods after hypophysectomy. Indeed, at 20 days after operation, no droplet formation after typhoid injection was observed (Table 3). However, it was difficult to be absolutely certain of this since the nucleoli, which stained like colloid droplets, were abundant in the nuclei which filled the flattened epithelial cells.

4. *Effect of Sera.* Rat sera were never toxic or lethal when administered to hypophysectomized rats by intracardiac injection. Of the rat sera tested, only those from certain of the thyroidectomized or thiouracil-treated normal rats stimulated droplet formation in the

PLATE 1. Thyroids of hypophysectomized male rats one day after operation and one hour after the intracardiac injection of the following materials. $\times 445$.

- FIG. 1. Untreated control.
- FIG. 2. 100 μ g. of hog pituitary extract in 0.5 cc. of water.
- FIG. 3. 1 mg. of histamine diphosphate in 1.0 cc. of water.
- FIG. 4. 1 cc. of typhoid vaccine.

Note absence of intracellular colloid droplets in thyroid epithelium in Fig. 1, and presence of droplets in Figs. 2, 3 and 4.

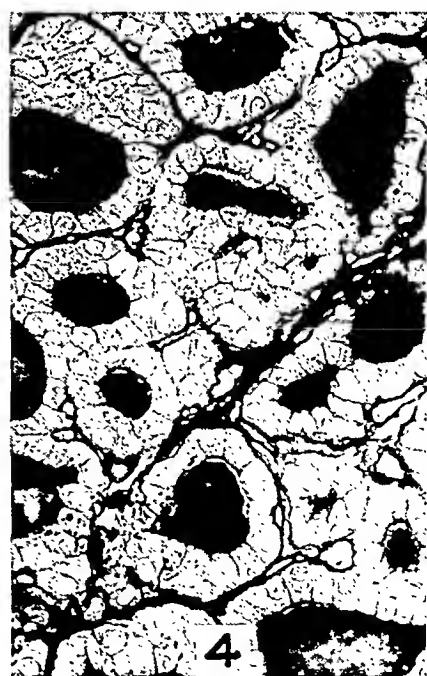
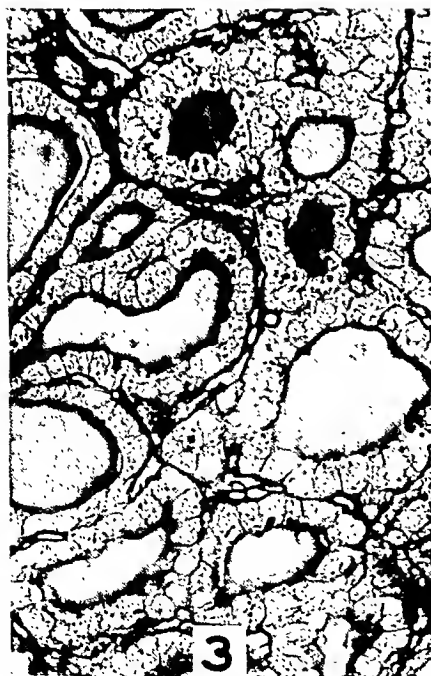
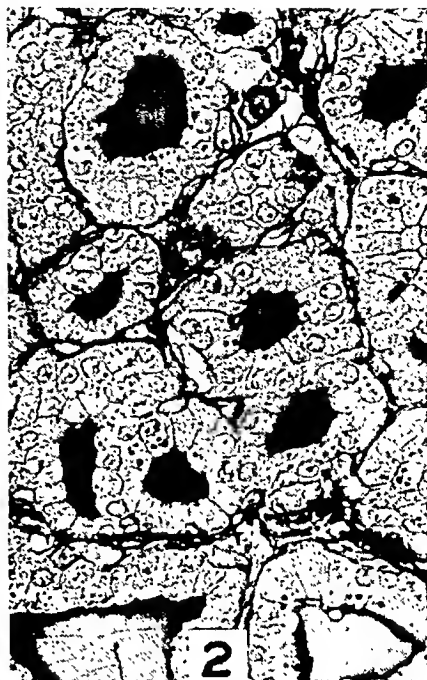
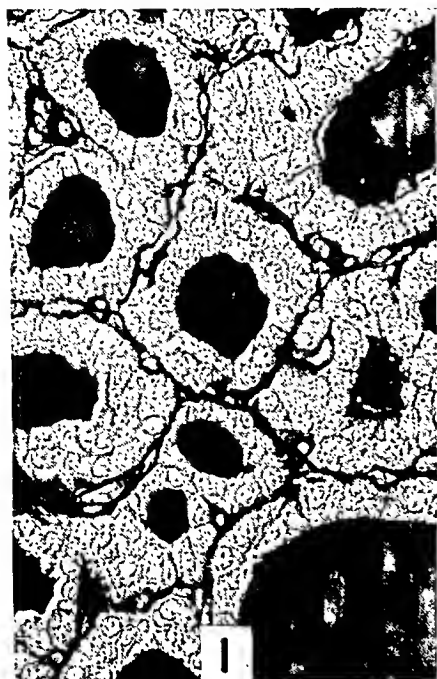


PLATE 1. See opposite page for explanation.

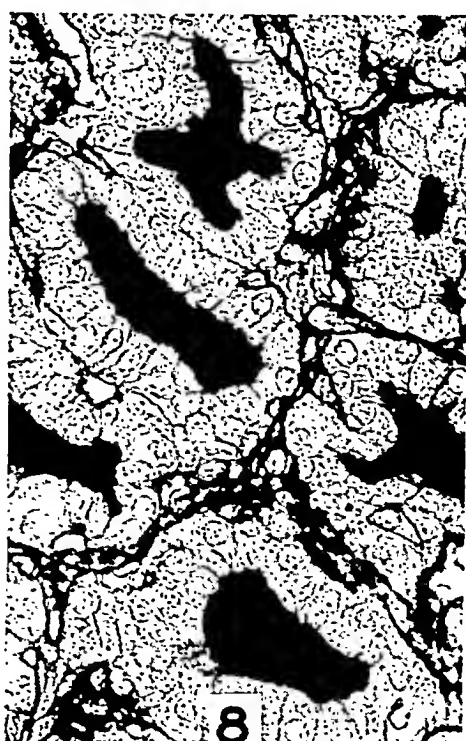
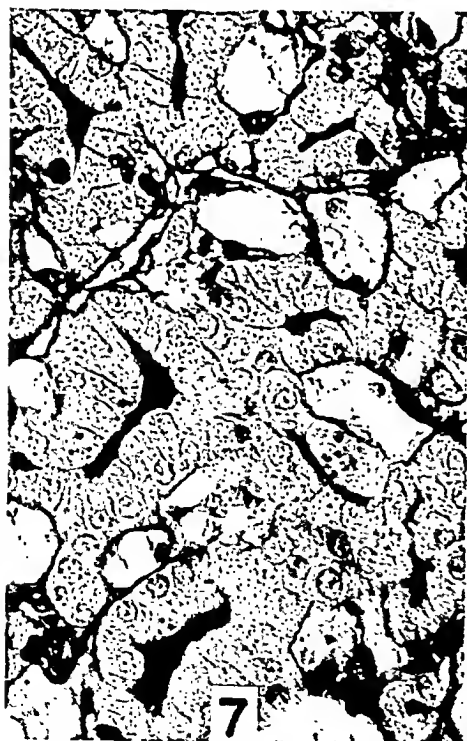
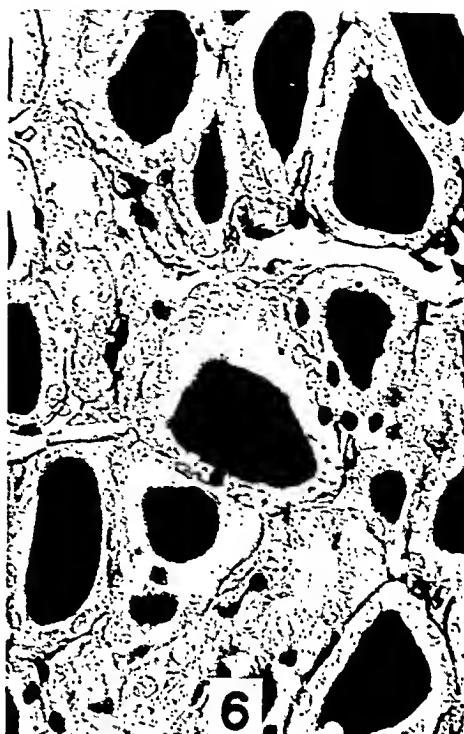


PLATE 2. See opposite page for explanation.

SPECIFICITY OF THYROTROPIN ASSAY

recipient hypophysectomized rats (Table 4). The serum from thyroidectomized rats housed at 5°C. for 17 hours following operation did contain a droplet stimulating material. If the rats were kept at room temperature their sera did not stimulate droplet formation in recipient rats. The serum from rats kept at room temperature for 10

TABLE 4. ASSAY OF SERUM OF THYROIDECTOMIZED OR NORMAL OR THIOURACIL-FED NORMAL YOUNG RATS FOR DROPLET STIMULATING MATERIAL. DONOR RATS WERE KEPT AT VARYING TEMPERATURES FOR VARYING TIMES. ASSAY WAS PERFORMED BY INTRACARDIAC INJECTION OF UNMODIFIED SERUM INTO HYPOPHYSECTOMIZED RATS ONE DAY AFTER OPERATION. ANIMALS WERE AUTOPSED ONE HOUR AFTER THE INJECTION

Type of animal	Serum assayed				Assayed on		Total no. of colloid droplets in 25 follicles	
	Animal kept Temp. °C.	For time	Vol. of pooled serum cc.	From animal #	No. of rats	Vol. inj. per animal cc.	Donor	Recipient
Normal	25							
Normal	5	16 hrs.	4	2	2	2.0	13, 50.	0.0.
Normal	5	16 hrs.	9	5	4	2.0	109, 117, 226, 279, 339.	0.0, 0.0, 0.
Normal ¹	5	16 hrs.	6 ¹	3	2	2.0	32, 149, 194.	0.0.
Thyr.	25	17 hrs.	8	4	3	2.0	12, 17, 63, 98.	0.0, 0.
Thyr.	5	17 hrs.	5	3	2	2.0		0.0, 0.
Thyr.	25	10 d.	4	2	2	2.0		0.0.
Normal	5					1.7		143, 158.
Thiouracil-Fed	25	7 d.	8	4	4	2.0	138, 146, 147, 175.	28, 36.
							135, 169, 177, 245.	

¹ 4 cc. serum mixed with 4 mg. thiouracil, and kept at 25°C. for 30 minutes prior to assay.
² Donor animals injected subcutaneously with 200 µg. Nal in 0.2 cc. at start and again midway through period of exposure to cold.

lays following thyroidectomy stimulated formation of small numbers of droplets in the thyroids of the recipient animals (Table 4). Although droplet formation was stimulated in the thyroids of normal rats exposed to cold, their sera did not stimulate droplet formation in recipient hypophysectomized rats. Nor did treatment of the donor normal cold-exposed rats with iodide permit such a material to be demonstrated. Nor did the serum of cold-exposed normal rats contain an agent which could be rendered active by incubation with thiouracil prior to injection into hypophysectomized rats. The serum from rats fed thiouracil for 7 days contained a droplet stimulating agent, and droplets were also present in the thyroids of the donor rats (Table 4). This was not due to the presence of thiouracil in such serum, since injection of thiouracil did not induce droplet formation in the thyroids of hypophysectomized rats (Table 2).

PLATE 2. Thyroids of young female rats. X 465.

FIG. 5. Exposed to 5° C. for 2 hours before autopsy.

FIG. 6. Fed. 0.1% thiouracil and treated with a 1.5 mg. pellet of mono-sodium d, l-thyroxine for 10 days. Note the intracellular colloid droplets in the follicles in the upper right hand corner of the figure.

FIG. 7. Fed 0.1% thiouracil for 10 days.

FIG. 8. Fed 0.1% thiouracil for 11 days, and hypophysectomized one day before autopsy.

Most of the human sera, and particularly those of post-operative patients, were toxic and even lethal to the test animals. Positive responses were frequent and seemed unrelated to the clinical status of the donor individual. Droplet formation was occasionally stimulated by injection of serum from normal individuals or from non-thyroid post-operative cases (Table 5). The numbers of droplets which formed seemed related to toxicity of the sera in the normal group. Although droplet formation was usually prominent following the in-

TABLE 5. INTRACELLULAR COLLOID DROPLETS OF THYROID EPITHELIUM OF ADULT RATS, ONE DAY AFTER HYPOPHYSECTOMY AND ONE HOUR AFTER THE INTRACARDIAC INJECTION OF 2 CC. OF SERUM FROM PATIENTS WITH THE FOLLOWING THYROID DISEASES

Diagnosis	Normal	Untreated hyper-thyroid	Iodine-treated hyperthyroid	Thiouracil-treated hyper-thyroid	Post thyroid-ectomy	Post operative (not thyroid-ectomy)
No. of Colloid Droplets in 25 Follicles Actual Results	0, 0. 0, 0. 0, 0, 0. 0, 0, 0. 0, 4. 0, 5. 16, 37, 56. 30, 51. 65, 70.	0, 0, 0, 12. 14, 15, 25, 73. 32, 60, 101.	0, 15, 22. 17, 19, 47. 7, 32, 40, 45, 115.	0, 20. 14, 26.	8, 9. 14, 29, 36. 31, 32, 40. 40, 78. 46, 75, 113, 172. 135. 121, 137, 147.	2, 4. 9. 45, 74.
Average No. of Droplets and Toxicity ¹	0 0 0 0 0 0 0 0 2 0 3 0 36 + + + 41 + + + 72 + + +	6 32 + + + 64 +	12 28 + + + 48 +	10 + 23 +	9 + + + + 28 + + + + 34 + + + + 59 + + + + 101 + + + + 135 + + + + 135 + + + +	3 + + + + 9 + + + + 60 + + + +

¹ 0 non-toxic.
+ slightly toxic.
++ somewhat toxic.
+++ toxic and lethal to some rats.
++++ lethal to all rats.

jection of the more toxic sera in the other groups, there were some sera which were very toxic and which stimulated the formation of but few droplets (Table 5).

5. *Effect of Hypophysectomy on Droplet Formation.* A group of 12 normal rats were exposed to 5°C. for 2 hours. At this time the left lobe of the thyroid of each animal was removed. These lobes contained an average of 70 droplets with a range of from 16 to 175. Hypophysectomy was performed at this time in half of these animals, and the pituitary glands were not removed in the other half. Exposure to cold was continued for an additional 16 hours. At the end of this time, the right lobe of the thyroid of each hypophysectomized animal was completely free of colloid droplets, while the thyroids of the animals with intact pituitary glands contained an average of 66 droplets with a range of from 23 to 147 colloid droplets.

Hypophysectomized rats, exposed to 5°C. for 2 hours one day following operation, did not form any colloid droplets in their thyroids (Table 6).

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TABLE 6. INTRACELLULAR COLLOID DROPLETS AND CELL HEIGHT OF THYROID EPITHELIUM OF YOUNG FEMALE RATS EXPOSED TO 5°C. FOR 2 OR 4 OR 16 HOURS. SOME COLD EXPOSED ANIMALS WERE PRETREATED WITH A SINGLE SUBCUTANEOUS INJECTION OF 100 μ g. NaI OR 10 μ g. d,l-THYROXINE. ONE GROUP WAS HYPOPHYSECTOMIZED (HYPOX.) AND ANOTHER GROUP WAS ADRENALECTOMIZED (ADRENX.) ONE DAY PRIOR TO COLD EXPOSURE. ONE GROUP OF NORMAL RATS, KEPT AT ROOM TEMPERATURE (25°C.), WAS INJECTED SUBCUTANEOUSLY FOUR TIMES, EACH AT $\frac{1}{2}$ HR. INTERVALS, WITH 0.05 CC. OF 1:1000 EPINEPHRINE HYDROCHLORIDE, BEFORE AUTOPSY. ANOTHER GROUP WAS INJECTED SUBCUTANEOUSLY TWICE, EACH AT HOURLY INTERVALS, WITH 1.0 CC. OF ADRENAL CORTICAL EXTRACT (A.C. EX.) BEFORE AUTOPSY

Treatment	No. of animal	Total no. of intracellular cold droplets in 25 follicles					Cell height— μ
		Av. $\pm \sigma$	Ratio control—1.00	P	P _t	Actual results	
Untreated Controls	13	8.4 \pm 23.2	1.00				Av. $\pm \sigma$
5°C.—2 hrs. Controls	7	248.2 \pm 127.1	29.55			0, 0, 0, 0, 0, 0, 1, 2, 3, 3, 16, 84.	7.19 \pm 0.54
5°C.—2 hrs. 100 μ g. NaI.	11	170.9 \pm 148.8	20.35	>0.3	<0.01	58, 105, 140, 221, 306, 349, 368.	8.10 \pm 0.36
5°C.—2 hrs. 10 μ g. thyrox.	12	17.9 \pm 42.6	2.13	<0.01	<0.01	0, 0, 0, 19, 37, 176, 206, 213, 305, 411, 513.	7.62 \pm 0.90
5°C.—4 hrs. Controls	7	96.0 \pm 104.8	11.43		<0.01	0, 0, 0, 0, 1, 1, 2, 2, 3, 3, 60, 143.	7.97 \pm 0.57
5°C.—4 hrs. 100 μ g. NaI.	6	80.0 \pm 52.1	9.52	>0.7		0, 12, 50, 116, 133, 141, 220.	7.49 \pm 0.75
5°C.—4 hrs. 10 μ g. thyrox.	7	0.7 \pm 1.5	0.08	<0.05	<0.01	7, 41, 70, 85, 103, 174.	7.83 \pm 0.55
5°C.—2 hrs. Hypox.	6	0.0 \pm 0.0	0.00			0, 0, 0, 0, 0, 1, 4.	7.00 \pm 0.67
Adrenx. Controls	4	21.5 \pm 29.4	2.56			0, 0, 0, 0, 0, 0.	6.97 \pm 0.37
5°C.—2 hrs. Adrenx.	4	194.0 \pm 88.4	24.05	<0.01	<0.01	0, 10, 11, 65.	8.00 \pm 0.46
Epinephrine 0.2 mg.	6	1.0 \pm 1.2	0.12			64, 217, 235, 260.	7.95 \pm 0.39
A.C. Extract 2.0 cc.	4	14.3 \pm 17.3	1.70			0, 0, 0, 1, 2, 3.	7.73 \pm 0.58
5°C.—16 hrs. Controls	8	180.6 \pm 99.1	21.50	<0.01		0, 0, 22, 35.	8.50 \pm 0.30
						32, 109, 117, 149, 194, 226, 279, 339.	8.20 \pm 0.31

P—P values are those comparing the group with the corresponding control cold exposed group.
 P—P values are those comparing the group with the corresponding NaI treated group.
 The control cold exposed groups are compared with the untreated controls.

A group of normal rats were fed a diet containing 0.1% thiouracil for 10 or 14 days. Their thyroids contained numerous colloid droplets in the tall thyroid epithelium (Table 7; Fig. 7). Hypophysectomy, performed after 10 days of thiouracil feeding in one group of rats, resulted in the complete disappearance of colloid droplets as early as one day after operation, despite continuation of thiouracil feeding (Table 7; Fig. 8). No new droplets formed during a 4 day period following hypophysectomy, despite continuation of thiouracil feeding (Table 7). Thyroid gland cell height and weight declined progressively following operation (Table 7).

6. *Effect of Thyroxine and Iodide Administration on Droplet Formation.* Exposure of normal rats to 5°C. for 2 or 4 or 16 hours resulted in a marked increase in the number of colloid droplets in the

The daily subcutaneous injection of 8.9 mg. of NaI in rats fed 0.1 thiouracil for 14 days did not reduce the number of intracellular colloid droplets or the cell height of the thyroid (Table 7). Thyroid weight, however, was reduced by about one-third.

7. *Are the Adrenal or Its Secretions Responsible for Droplet Formation?* Small numbers of colloid droplets were present in 3 of 4 adrenalectomized animals kept at room temperature one day after operation. Adrenalectomy did not prevent an increase in the number of droplets after exposure to 5°C. for 2 hours (Table 6).

Treatment of normal rats kept at room temperature with injections of epinephrine or adrenal cortical extract did not stimulate formation of intracellular colloid droplets (Table 6).

DISCUSSION

Under some conditions, the formation of colloid droplets in the thyroid epithelium of living rats was related to activation by pituitary extract or dependent on the presence of the pituitary gland. Thus, droplet formation in the thyroid of the hypophysectomized rat occurred promptly following the administration of small dosages of pituitary extract. Also, hypophysectomy resulted in the complete disappearance of colloid droplets from the thyroids of animals in whom they had been formed by cold-exposure or thiouracil feeding. Nor did droplets form in the thyroid of hypophysectomized rats after exposure to cold or after thiouracil administration. Further evidence that the formation of droplets in the thyroids of cold-exposed or thiouracil-fed normal rats was mediated through the pituitary is provided by the experiments in which thyroxine, but not iodide, was shown to inhibit this response. This effect of thyroxine was probably due to an inhibition of release of thyrotrophic hormone of the pituitary gland rather than to an interference with the response of the end organ. Thyroxine did not interfere with the response of the hypophysectomized rat to simultaneously administered pituitary extract, but did inhibit the formation of droplets in normal rats exposed to cold or fed thiouracil. This inhibition was apparent as early as two hours after the injection of thyroxine.

The response of the thyroid to cold-exposure was not mediated through the adrenals since it occurred in adrenalectomized rats. Nor was the stimulation of the adrenal cortex by exposure to cold (Long, 1947; Sayers and Sayers, 1947) responsible for the changes in the thyroid. In the dosages used, injections of either adrenal cortical extract or of epinephrine, which is secreted in increased amounts during cold-exposure (Cannon, et al., 1927; Cramer, 1928), did not stimulate droplet formation.

However, droplet formation was not a specific response for thyrotrophic hormones. Droplets formed in the thyroids of hypophysectomized rats following the injection of toxic dosages of typhoid

vaccine or histamine or pilocarpine. Although the reason for it is not clear, this response may be analogous to the spontaneous formation of these structures in vitro in surviving ischemic thyroid tissue (Champy, 1915; Demuth, 1932; Dvoskin, 1947d). Possibly, an ischemia of the thyroid gland in vivo may result following the injection of toxic agents. Or, possibly, these agents may have a chemical irritant effect on thyroid tissue somewhat akin to the effect of mechanical irritation in stimulating droplet formation (Williams, 1944; Dvoskin, 1947d). Whether proteolytic enzymes or inactivated adherent thyrotrophic hormone became activated under these conditions remains undetermined.

The decrease in response to pituitary extract and typhoid vaccine as the interval after hypophysectomy was increased is of interest. Whether this represents a true decrease in sensitivity, or signifies a delay in response to these materials at one hour after injection, with a possible peak response at some later time, is not known. Theoretically, the hypophysectomized rat at 20 days after operation might provide a suitable test object for thyrotrophic hormone, since the non-specific response to typhoid did not occur at this time. However, such an animal responds less to pituitary extract at this time. Also, the counting of droplets is complicated by the paucity of cytoplasm and the plethora of nucleoli, which stain like colloid droplets, in the prominent nuclei of the flattened thyroid epithelium.

The fact that toxic materials may stimulate droplet formation in the thyroid of the hypophysectomized rat one day after operation could explain the disparity of results obtained in attempts to assay the thyrotrophic hormone content of rat as compared with human sera.

Rat sera were never toxic or lethal to the test animal. Even the sera of rats following thyroidectomy were non-toxic. With rat sera, no positive response was ever obtained unless the animal had been thyroidectomized or fed thiouracil. In the presence of an intact thyroid, no positive test was obtained, even after the donor animal had been exposed to cold. Possibly the inactivation of circulating thyrotrophic hormone by the thyroid (Seidlin, 1940; Rawson, Sterne and Aub, 1941, 1942) may explain the absence of detectable amounts of this material in our assay of sera from intact rats. No detectable activity was found in the serum of rats thyroidectomized for short periods. However, thyroidectomy followed by exposure to cold for a short period resulted in the presence in the serum of large amounts of a material stimulating droplet formation. The positive responses secured with serum from normal thiouracil-fed rats is in agreement with the results of Grasso and De Robertis (1946), who used intact rats for assay purposes. Possibly, this is due to the reactivation (Rawson, Albert, McArthur, Merrill, Lennon and Riddell, 1946) or augmentation (Albert, Rawson, Merrill, Lennon and Riddell, 1947)

of thyrotrophic hormone by thiouracil. However, incubation of serum from normal rats exposed to the cold with thiouracil did not result in a positive response in our experiment.

Human sera, on the other hand, were frequently very toxic and lethal to the test animals. Positive responses were also very frequent and were unrelated to the thyroid status of the individual. Among the normal subjects, the positive responses seem related to the toxicity of the sera. Among the other groups, this relationship was less clear-cut. Some of these sera were very toxic, and yet induced but little droplet formation.

Since droplet formation may be induced in hypophysectomized animals with toxic dosages of typhoid vaccine or histamine or pilocarpine or with toxic human sera, though admittedly we may have a pituitary factor present in the case of the sera, one must regard the results of such assays for thyrotrophic hormone using droplet formation with suspicion. This is particularly true when rats with intact pituitary glands are used (Grasso, 1946; Grasso and De Robertis, 1946) since in addition to the possibility of a non-specific response, one has the added possibility of participation of the test animals' own pituitary gland.

CONCLUSIONS

Under some conditions, the formation of colloid droplets in the thyroid epithelium of living rats was related to activation by thyrotrophic hormone. In hypophysectomized rats, the administration of small dosages of pituitary extract was followed by a prompt appearance of large numbers of droplets. Larger dosages stimulated formation of proportionately larger numbers of droplets. This response was not prevented by the simultaneous administration of thyroxine or iodide. There was a progressive decrease in the number of droplets which formed one hour after the injection of a fixed dosage of pituitary extract as the interval after hypophysectomy was increased. The droplets present in the thyroid epithelium of normal rats exposed to cold or fed thiouracil promptly disappeared following hypophysectomy. Droplet formation was inhibited in normal rats exposed to cold or fed thiouracil by the simultaneous administration of thyroxine, but was not inhibited by administration of iodide. The adrenal or its secretions were not responsible for droplet formation.

Under other conditions, droplet formation was independent of thyrotrophic hormone since droplets appeared in the thyroid epithelium of hypophysectomized rats one day after operation and one hour after the injection of toxic dosages of typhoid vaccine or histamine or pilocarpine. Droplet formation at one hour after typhoid injection was markedly decreased at 8 days after hypophysectomy, and was absent at 20 days after hypophysectomy.

Fresh human sera were often toxic and lethal when injected into hypophysectomized rats. The numerous positive responses which re-

sulted from these injections were unrelated to the thyroid status of the donor.

Fresh rat sera were never toxic or lethal. Positive responses were obtained with sera from thyroidectomized rats or thiouracil-fed rats, and were not obtained under other conditions in the presence of an intact thyroid gland in the donor rat.

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ENDOCRINOLOGY

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COMB-TESTIS RELATIONSHIP

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IT HAS been known for some time that when cockerels are caponized, the comb regresses. Also when an androgenic substance is applied topically to the comb of a capon, or when injected intramuscularly, the growth of the comb is resumed. This has been made the basis of a quantitative measure of the androgenic activity of crystalline androgens, and tissue and urine extracts. Later, it was shown by Buchner *et al.* (1934), Hoskins and Koch (1939), Marlow and Payne (1940) and Zowadowsky (1935) that the removal of the comb results in significant increase in the size of the testis of a cockerel.

There would then appear to be a reciprocal relationship between the comb and testis. Whether or not this reciprocity also includes the pituitary has not been established. Zowadowsky (1936) upon feeding ground cockerel's combs and injection of impure alcoholic extracts in male rats reports a smaller testis, seminal vesicles and prostate glands. Hoskins and Koch (1939) were not able to confirm Zowadowsky's results but on the contrary noted a slight but not a mathematically significant increase in the size of above-mentioned organs. They further tried the same experiment on fowls which perhaps should be more receptive to dried ground cockerels' combs in the diet than rats and again noted slight but not significant increase in body weight, testis weight to body weight ratio, and in comb growth. Feeding of dried comb tissue to capons failed to show an active substance. No inhibitory substance could be found.

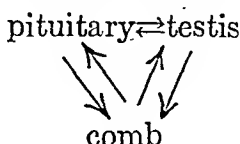
Landauer (1942) in a series of experiments obtained testis growth by several methods: Removal of wattles, comb and wattles, and the posterior half of the comb gave increased testicular weights, but doubtful or negative results were obtained when the combs alone were removed, edges of comb or wattles clipped, or horizontal slits made in the comb. Zowadowsky explained his results on a reciprocal basis which may be expressed as below.

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pituitary \rightleftharpoons testis \rightarrow comb

Koch went one step further and stated that the interrelationships between the three organs were perhaps better expressed by:



but suggested that further work must be done to show the involvement of the pituitary. Landauer's (1942) explanation is that there is no endocrine interrelationship but that the results noted were caused by trauma.

In an effort to aid in clarification of this problem, the author outlined an experiment removing the comb from baby chicks 3 days old in which trauma certainly would be little or negligible. The combs were removed by scissors with little or no loss of blood. It is believed that this treatment to young chicks would reduce traumatic shock to a minimum or eliminate its effect. No work has been reported on baby chicks in this regard as most birds were used at 21 days or over when cutting the combs produces quite a traumatic shock, although it is difficult to see how the effects could continue over a period of several months. Purified comb extract was used on dubbed birds and on normal cockerels, and its effect studied on the growth of the testis, comb, wattle and body. Young and old, male and female rats were also included. Observations on the vaginal smear of the older female rats were made and vaginal introitus of the young female rats studied. Weight of seminal vesicles and testis of both old and young male rats were obtained.

EXPERIMENTAL

Preparation of Experimental Animals

The combs of single comb, white leghorn cockerels three days old were removed by applying a pair of scissors to the posterior portion of the comb and snipping the comb off to the beak close to the head. Very little bleeding, and in many cases none, was noted. If left alone a rosette of comb material grows back slowly. To minimize this a saturated solution of trichloroacetic acid was placed on the wound and renewed growth was minimized, but not prevented with the one application.

Preparation of Purified Extract

The combs from single comb white leghorn cockerels of broiling age which were removed soon after death, were ground with a meat chopper and extracted with 95 per cent alcohol in a continuous extractor (1935). This extract was chilled overnight to remove neutral fats. The alcoholic extract was evaporated to dryness by distilling under reduced pressure. Absolute

alcohol was added in several portions and removed under suction, thus removing final traces of water. This was then dissolved in the least quantity of absolute alcohol possible and chilled to remove toxic sterols, and remaining neutral fats. The alcoholic solution was again evaporated to dryness as above. The residue was dissolved in ether and 4 volumes of acetone added to remove phospholipins. The ether-acetone solution was then evaporated by distillation and a calculated amount of corn oil (Mazola) added, just before all of the solvent was removed. Another portion of the ground comb tissue was extracted thoroughly with 0.9 per cent saline. This saline solution was adjusted to a pH of maximum flocculation of protein (pH 4.14). This protein residue was centrifuged out, taken up in 0.9 per cent saline and adjusted to pH of 7.33.

Injection of the purified alcoholic extract in oil or the neutral saline emulsion in rats showed none of the toxic effects reported by Zowadowsky (1935-36) which produced, according to reports, decreased growth, decreased size of testis, etc. Daily injections were carried out over periods of 10 days or more without toxic symptoms.

Histological examination of the testis, combs, uteri and ovaries of birds treated with comb extracts show no degenerative effects when compared to saline injected controls.

RESULTS OF ADMINISTRATION OF SALINE EXTRACT

A dosage equivalent to $3\frac{1}{2}$ gm. of comb tissue was injected daily for 10 days in old and young normal female and in young male rats. No disturbance of the estrus cycle of the females could be detected, and no effect of vaginal introitus of young females were noted. Ovarian weight/body weight and weight of uterus to body weight ratios of the females showed no calculated significant differences over controls. Weight of seminal vesicle to body weight and weight of testis to body weight changes were not significantly different.

ADMINISTRATION OF PURIFIED ALCOHOLIC EXTRACT

A dosage equivalent to 10 gms. of comb tissue was injected into young and old female and young male rats with similar negative results as cited above (Table 1). It is expected that the genitalia of cocks would more likely be affected by comb tissue than those of rats as suggested by Hoskins and Koch (1939). The purified alcoholic extract dissolved in corn oil (Mazola) was spread on the head furnishing of cockerels by use of a 1 cc. hypodermic needle following generally the procedure of Frank *et al.* (1942). Four pens of fowls were prepared:

1. Pen #1 contained 25 baby chicks from which the comb had been removed and alcoholic extract of comb in Mazola applied daily. (Dosage equivalent to 0.69 gm. fresh comb tissue daily per fowl was administered.)
2. Pen #2 containing 25 baby chicks from which the combs had not been removed and alcoholic extract of comb tissue in corn oil

(Mazola) applied daily. (Dosage equivalent to 1.15 gm. fresh comb was used daily.)

3. Pen #3 containing 25 baby chicks from which the combs had been removed but corn oil (Mazola) administered daily.

4. Pen #4 containing 25 chicks in which the combs had not been removed but corn oil (Mazola) added daily.

The combs were removed from chicks in pens 1 and 3 at 3 days of age, and the alcoholic extracts applied to the comb or rosette for a period of 38 days twice daily. The feathers had been removed by scissors in the vicinity of the comb so as to minimize loss of material

TABLE 1. EFFECT OF EXTRACTS OF COMB TISSUE ON RAT GENITALIA

Treatment	Body weight (av.)	Females (Response)			
		Uterine weight (av.)	Ovarian weight (av.)	Uterus (mg.)	Ovaries (mg.)
				Body weight (gm.)	Body weight (gm.)
Oil	62	62	26	1.00	.42
Comb extract	57	52	23	.91	.40
Saline	49	47	27	.96	.56
Saline extract	43	32	17	.74	.40
Males (Response)					
		Testis weight	Seminal vescl. weight	Testis (mg.)	Sem. vescl. (mg.)
				Body weight (gm.)	Body weight (gm.)
Oil	49	563	14	11.50	.29
Comb extract	56	611	16	10.90	.29
Saline	56	596	13	10.60	.21
Saline extract	49	588	15	12.00	.30

on the feathers. The total amount of alcoholic extract or oil administered to the combs of the fowls in a pen for a given number of fowls was recorded for the entire period rather than an individual record of each chick. At the end of this time the chicks were fasted for 36 hours to empty the gut, and then killed. Combs, wattles and testis were removed and weighed. The average result of these weights is recorded in Table 2.

It is observed that on the basis of average figures that the testicular weight of fowls receiving the extract on a normal comb and on the fowls with comb removed, and receiving corn oil (Mazola) only, were identical, while the one receiving the extract on the rosette was greater than either. This latter suggests an additive effect of removal of comb and application of the extract. It is not strictly proportional due first perhaps to less surface for absorption of the extract on the normal comb rather than on the rosette and secondly in this experiment to a difference in daily dosage (0.69 gm. equivalent of fresh tissue for Pen #1 and 1.15 gm. equivalent of fresh tissue for Pen #3). Calculations on this basis indicate that the effect of removing the comb alone on the testis to body weight ratio would be .08, while the

COMB-TESTIS RELATIONSHIP

TABLE 2. EFFECT OF PURIFIED COMB EXTRACT ON CHICK COMB, WATTLES AND TESTIS

Pen No.	No. animals	Initial body weight	Body weight (av.)	Comb weight (av.)	Wattle weight (av.)	Testis weight (av.)	Ratios to body weight		
							Comb (av.)	Wattle (av.)	Testis (av.)
1	24	43.5 g	316	.399 g*	.392 g	.160 g	1.22	1.25	.51
2	26	41.1 g	324	3.504 g	.456 g	.139 g	10.56†	±.13	±.06
3	21	45.0 g	310	.430 g	.286 g	.126 g	±.01	1.36	.43
4	24	41.5 g	299	2.072 g*	.327 g	.085 g	1.30*	±.19	±.04
							6.89	±.10	±.04
							±.01	1.08	.28
								±.01	±.02

* Rosette is a growth of comb after removal.

† Cannot be computed as combs were removed and some were treated with concentrated trichloroacetic acid and growth was hence irregular.

extract gives an added .15 increase over the normal effect. If both pens had received the larger quantity (1.15 gm. daily) the average testicular weight could be calculated at .59 instead of .51 maintaining a significant difference. Table 3 shows the calculated "t" values for this experiment.

The fowls in pens 2 and 4 from which the comb has not been removed are the only ones for which a "t" value for comb differences could be evaluated. Using Fisher's (1938) tables of "t" values the increase in size of the combs of the chicks in pen #2 receiving the comb extract over the ones in pen #4 receiving oil only is highly significant. The increase in wattle growth was not as significant as the comb growth. The ratio of organ weight in milligrams over the body weight in grams was used throughout in order to equalize the size relationships. Body weights of the fowls treated with the comb extract or the controls receiving oil alone does not show a significant difference.

ASSAY OF ANDROGENIC SUBSTANCE

Twenty-five 2 day old single comb white leghorn cockerels were treated with the alcoholic extract of comb as indicated in an earlier

TABLE 3. COMPARATIVE "T" VALUES OF COMB, WATTLES AND TESTIS OF CHICKS

Pens	Ratio to body weights			Testicular weights	
	Combs "t" value	Wattles "t" value	Testis "t" value	"t" value	"t" significant value
1&2	*	.7015	.1013	.7271	2.660
1&3	*	2.116	.1064	1.411	2.660
1&4	*	1.095	.3.803	3.911	2.660
2&3	*	2.273	†	.6001	2.660
2&4	2.645	1.854	2.942	3.193	2.660
3&4	*	.1143	3.372	2.422	2.660

* Could not be compared as comb had been removed from birds in pens 1 or 3.

† Could not be calculated as the difference of their means is zero.

paragraph. Fifteen were treated with a known quantity of androsterone acetate. Ten received corn oil (Mazola). This was applied to the comb with a 1 cc. Luer syringe. To minimize the spreading of the oil or extract to the feathers, a commercial hair remover was applied around the comb and on top of the head to remove downy feathers. After standing some ten minutes, the hair remover was washed off carrying with it all the fine hair or feathers on the head of the chick. This allowed a clean head with little danger of the oil spreading to any feathers.

To prevent too wide spreading of the vehicle over the head near the comb a divided daily dosage was applied twice daily. A maximum average dosage of .01 to 0.2 ml. was applied, as any larger volume

TABLE 4. ASSAY OF ANDROGENS

Pen No.	No. animals	Body weight		Comb weight	Comb		Size	Testis weight	Ratio (mg.)/Body Wt. (gm.)	
					L	H				
		Int.	End	(av.)	(av.)	(av.)			Comb	Testis
		gm.	gm.	mg.				mg.		
1*	23	40	64	34	10.8	3.6		18	.52	.26
2†	15	40	62	79	11.8	4.4		15	1.27	.26
3‡	10	40	65	29	10.3	3.5		19	.44	.29
										11.8
										37.0
										8.5§

* 1 = on Extract of comb; † 2 = Androsterone-acetate; ‡ 3 = Mazola.
 § A personal communication from one of the authors (E.K.) whose method for assay of androgens by the chick method (8) implies that in their hands #3 above would have a demg. of zero.

(.05 ml.) spread too much over the head. This was continued for seven days and on the 8th day the chicks were sacrificed. The comb and testis were removed and weighed, and the comb measured. The differences in the weights of the wattles were too small to be significant. Each experimental chick received an average total dosage for the seven days equivalent to 11.5 gm. of fresh comb tissue. The control chicks received an average of 13.5 micrograms of androsterone acetate over the same period. It is very evident from Table 4 that the body weight changes were insignificant. It is clear that the comb had begun to increase, with only a slight trend in decreased weight of the testis noted. Seven days may be too short a time with this dosage for critical evaluation.

Calculations according to the method of Frank and others (1942) indicates that the comb extract contained 11.8 demg. (chick) as compared to 37.0 cdmg. of the androsterone acetate. This would seem to indicate that both the short time and the concentration of the extract was too little to give mathematically significant figures.

DISCUSSION

This increase in testicular weight when the comb is removed parallels closely the results of all other investigations except that of

Landauer (1942) who gets significant increase in testicular weight only if both comb and wattles are removed, and not when the comb alone is removed.

Contrary to the report of Zowadowsky (1935), but in slight agreement with Hoskins and Koch (1939), injection or topical application of an oil containing an alcoholic extract to the comb, increases the size of the comb and testis. None of the fowls were fed dried comb tissue, but the more concentrated non-toxic extract was used by topical application.

An androgenic assay conducted according to the method of Frank *et al.* (1942) using 3 day old baby chicks was made and an increase in the size of comb over that of control was noted. Androsterone acetate was applied in oil as a reference substance.

CONCLUSIONS

Removal of the combs of chicks 3 days of age results in a significant increase in the size of the testis, but not a significant increase in the size of wattles.

Topical application to the comb of a chick of a purified alcoholic extract of rooster combs resulted in an enlargement of the testes and combs over that of controls.

Quantitatively assay of the alcoholic extract indicates the presence of an androgenic compound of unknown composition, which may be effective through the pituitary, but it is not present in sufficient quantity in the dosage applied to alter the action of the pituitary. Berdnikoff and Chauncy (1935) obtained some androgenic activity upon hydrolysis of combs with hot concentrated trichloroacetic acid.

Using baby chicks as subjects where little trauma is encountered in dubbing, it is believed that there is some effective androgenic substance in the comb of roosters. Landauer (1942) did not get significant results with the removal of comb, clipping the comb, or slitting the comb, although the removal of both combs and wattles, wattles alone, or removal of posterior half of the comb was effective. Trauma would be encountered in all events.

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THE EFFECT OF INANITION ON THE INACTIVATION OF ESTROGEN BY THE LIVER¹

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IT HAS been established that exogenous estrogens can be inactivated by the animal organism with great facility. Zondek and Sklow (1941) have shown that only 5 per cent of the estrogen administered to rats can be recovered from the animal body three hours after its subcutaneous injection. Heller (1940); Singher *et al.* (1944) have demonstrated by in vitro studies that the liver is the organ involved in this degradation mechanism. Talbot (1939), Schiller and Pincus (1944) and others have shown an impairment of inactivation in the presence of hepatic parenchymal damage in the rat and Glass, Edmondson and Soll (1940) have demonstrated the same phenomenon in patients with advanced hepatic cirrhosis.

Golden and Sevringhaus (1938) implanted ovaries into a site drained by the portal vein and discovered that the rats showed no sign of estrus as determined by vaginal smear. However, if these ovaries were re-implanted into the axilla, estrus re-appeared. Biskind and Marks (1939) demonstrated a similar phenomenon with pellets of estrone implanted into the spleen. Biskind and Biskind (1942) carried out further studies to show that when rats were placed on a diet deficient in components of the vitamin B complex the liver lost its ability to inactivate the estrogen. These studies were confirmed and it was demonstrated that the important components of the B complex were thiamine and riboflavin (Segaloff and Segaloff, 1944). In the absence of either dietary thiamine or riboflavin the degradation mechanism of the liver for estrogens was impaired.

Drill and Pfeiffer (1946) have presented data to question the role of vitamin B in the inactivation mechanism of estrogens in rats, and showed that inanition is a much more important factor. Segaloff, however, previously had attempted to rule out the factor of inanition. It appeared profitable to reinvestigate this problem and attempt to separate the influence of the two factors, namely, vitamin B deficiency and inanition.

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METHODS AND MATERIALS

Adult female rats of the Long-Evans strain weighing approximately 200 Gm. were ovariectomized; 14 days later pellets of α -estradiol (1-3 mg.) were implanted under the capsule of the spleen. After a period of 14 days when the vaginal smears were of the diestrus type, the animals were placed on a vitamin B deficient diet (Drill and Pfeiffer, 1946) and allowed to eat ad libitum. The daily consumption of food was recorded.

Two groups of three rats were housed in each metabolism cage. Thiamine determinations were performed by a modification of the thiochrome method (Lowry, Bessey and Davis, 1948) on 48 hour specimens of urine collected in Erlenmeyer flasks containing 0.5 ml. glacial acetic acid. The animals were weighed at various intervals and vaginal smears were taken daily. The same six animals were studied through: 1) a period of vitamin B deficiency; 2) a period of restricted food intake with adequate vitamin B; 3) a period of unrestricted food intake and sufficient vitamin B.

At the conclusion of the experiment the animals were sacrificed, the pellets were removed from the spleen, cleaned, dried and weighed. The amount of estradiol absorbed averaged 12 micrograms/day/rat.

RESULTS

During the 31 days in which the animals were on a diet deficient in the components of the vitamin B complex (Figure 1, A) they lost an average of 28.2 per cent of their initial body weight. The food intake fell from approximately 40 Gm./day/3 rats to 10 Gm. The rats exhibited the physical manifestations of vitamin B deficiency. Thiamine excretion quickly fell to less than 1 microgram/48 hours/3 rats, which is an indication of decreasing thiamine stores (Holt, 1943). After approximately 18 days on this diet, the vaginal smears were continually of an estrus type.

The period of inanition with sufficient vitamin B consisted of 8 days (Figure 1, B) in length during which time the animals were fed 10 Gm./day/3 rats (an average of the previous week's daily food intake). In addition they were injected with 0.05 ml. Solu B.² Each animal continued to lose an average of 4 Gm. of body weight for the 8 day period. They appeared to be in better physical condition, however, and were much more active and quite vicious. The urinary excretion of thiamine rose to levels as high as 50 micrograms for a 48 hour period. However, the vaginal smears continued to be of the estrus type.

After this 8 day period of restricted food intake the animals were allowed to feed ad libitum (Figure 1, C) the same diet to which the components of the vitamin B complex had been added (Drill and Pfeiffer, 1946). The food intake quadrupled overnight; however, the thiamine excretion decreased due to diminished thiamine intake. The animals regained approximately 50 per cent of the weight lost during

² Each 0.05 ml. Solu B contains 50 γ thiamine, 50 γ riboflavin, 25 γ pyridoxine, 250 γ calcium pantothenate and 1.25 mg. nicotinamide.

the thiamine deficient period. Within 5 days after the institution of this diet, the vaginal smear began to revert to the diestrus type. A typical experiment is represented in Figure 1. The data for the other animals are practically identical with the one illustrated.

DISCUSSION

From the data presented, it appears possible to separate the effects of vitamin_B deficiency and the concomitant effects of inanition. In

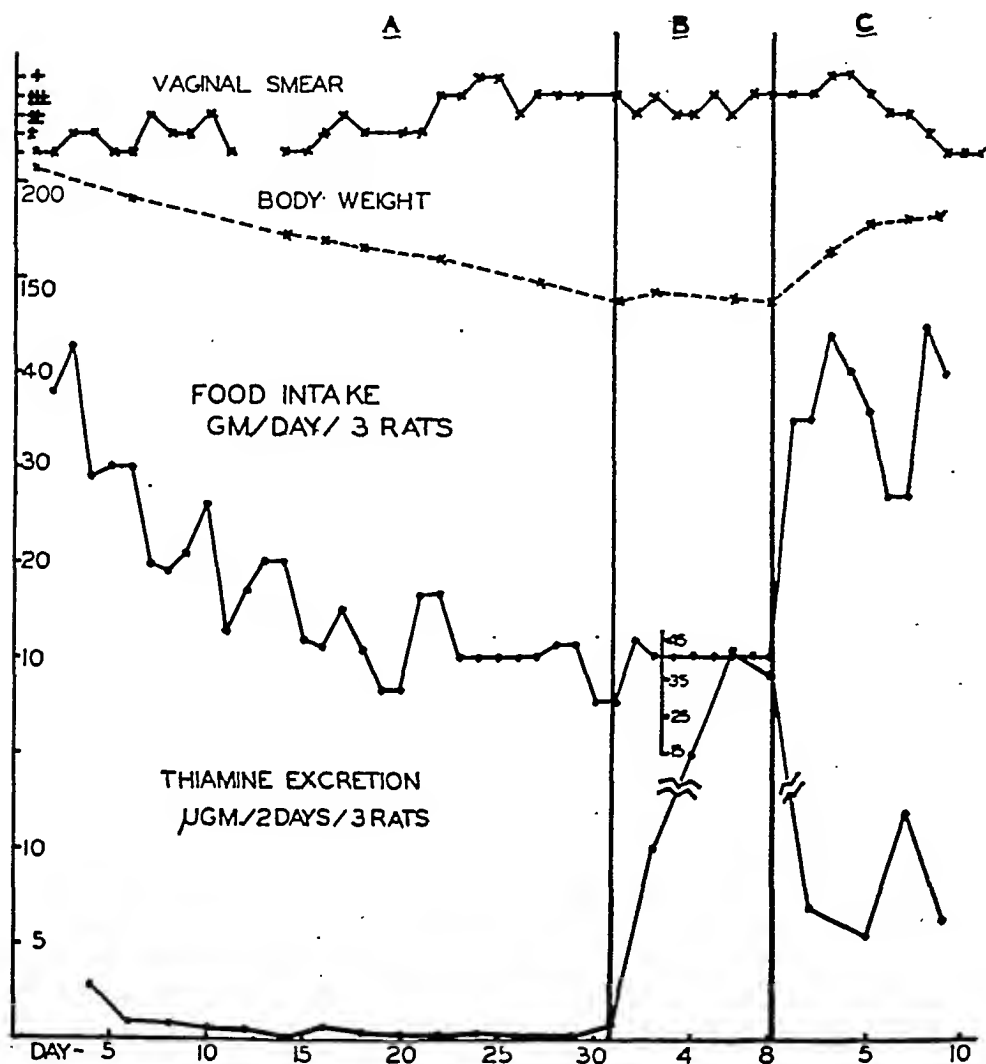


FIG. 1. A = Period of vitamin B deficiency; B = Period of restricted food intake plus vitamin B administration; C = Period of unrestricted food intake and sufficient vitamin B. The vaginal smears and body weights are of one representative animal of the group. The food intake and thiamine excretion are the total of three. The classification of vaginal smears is that employed by Kahnt and Doisy (1928):

- + Cornified smear
- ± ± ± Cornified + epithelial cells
- ± ± Epithelial cells
- ± Leucocytes + epithelial cells

spite of massive quantities of vitamin B, the rats were unable to inactivate estradiol when the diet was restricted. However, on the resumption of ad libitum feeding during which time the rats began to regain part of the weight lost, estrogen inactivation appeared.

Biskind *et al.* and Segaloff claim that it is the vitamin deficiency which interferes with the degradation mechanism, although their experiments were not well controlled for caloric intake. György (1945) has shown that hepatic damage caused by a low protein diet will result in the same phenomenon.

It is entirely possible that several factors are involved to cause the liver to lose its ability to inactivate estrogens. Protein deficiency and inanition have been shown by György, by Drill and Pfeiffer and in this study to be one of these factors. In the work of Biskind *et al.* and Segaloff, it should be noted that on the vitamin deficient diet the vaginal smears changed to the estrous type after only a few days (4-8), while in this experiment approximately 21 days were necessary to reach the estrous state. This dissimilarity may be due to differences in the strain of rats used. It is improbable, however, that it is due to differences in the synthesis of thiamine and riboflavin because these rats could not degrade estrogens when on restricted caloric intake even though the diet was supplemented with large amounts of vitamin B complex.

SUMMARY

Spayed female rats with pellets of α -estradiol implanted in the spleen and on a vitamin B deficient diet show no signs of estrus, as evidenced by vaginal smear, until they have lost approximately 20 per cent of the body weight. In spite of the administration of large amounts of thiamine and riboflavin, the vaginal smears remain of the estrous type, when the food intake is restricted. However, when the rats are then allowed to eat ad libitum, the liver regains its ability to inactivate estrogen. It appears that it is not deficiency of the B vitamins but the concomitant inanition which impairs the degradation mechanism for estrogens in the rat liver.

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EXCRETION OF 17-KETOSTEROIDS BY MALE RABBITS DURING ALTERED GONADAL FUNCTION

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By STANDARDIZATION of environmental conditions and selection of animals for such variables as health, weight, age, nutrition, it is possible to control factors which have been indicated as influencing the excretion of 17-ketosteroids (Landau et al., 1948; Forbes et al., 1947). Proper use of such controls should provide opportunity for critical investigations of 17-ketosteroid metabolism.

Relatively few studies have reported the urinary 17-ketosteroid excretion in laboratory animals. They have been assayed in the rat (Lampton and Miller, 1941), mouse (Karnofsky et al., 1944), chimpanzee (Dorfman et al., 1947), and rabbit (Kimeldorf, 1948). Unfortunately, values reported by these investigators are not strictly comparable because of differences in assay techniques.

The purpose of the present investigation is to study alterations in 17-ketosteroid excretion experimentally induced in rabbits whose normal excretion level has been established.

MATERIALS AND METHODS

Data reported in these investigations were derived from the study of 20 New Zealand Albino male rabbits. All were sexually mature and weighed 9.5-12.5 pounds with only three animals weighing more than 11.5 pounds. They were fed complete rabbit pellets and received water in heavy stone crocks to avoid urine dilution.

The methods used to collect urines, fractionate the extracts, and assay have been described in detail (Kimeldorf, 1948). Briefly, crude neutral steroid ether extracts of urine were fractionated with Girard's Reagent T by the micro-method of Pincus (1945). Resultant ketonic fractions were assayed by the Talbot et al. (1940) anhydrous-Zimmerman reaction. Assays were read by the aid of an Evelyn photoelectric colorimeter calibrated with androsterone and dehydroisoandrosterone. The quantity of 17-ketosteroids was determined for total 48 hour volumes of urine excreted by individual animals. Each urine was assayed in triplicate and standards were run with nearly all series of assays.

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TABLE 1. AVERAGE EXCRETION OF URINARY 17-KETOSTEROIDS (MG./48 HOURS) FOR EACH CONDITION INDICATED

	No. of collections	Total 17-KS	S.E.
Normal	109	2.31	0.10
Castrate	33	1.35	0.10
Cryptorchid	17	1.40	0.07
Cryptorchidectomized	11	1.29	0.29
Pituitary-injected	12	1.38	0.22
Testosterone-implanted	13	3.07	0.27

EXPERIMENTAL PROCEDURES AND RESULTS

Casteration. Ten animals were castrated to determine the total 17-ketosteroid excretion of rabbits in the absence of testicular secretions. The normal excretion of each animal was determined previously so that quantitative changes with castration could be more accurately evaluated. Following castration the animals were allowed three weeks recuperative period. Thirty-three urines of 48 hour duration were collected and assayed.

The mean excretion level of animals in the castrate condition was 58% of the mean excretion level of normal animals. Table 1 summarizes these data. A comparison of the two excretion levels for individual animals is presented in Table 2 and indicates an average decrease of 41% with castration.

Animal B castrate excretion of 17-ketosteroids was determined under different conditions than those of other castrates. The excretion level of this animal was determined seven months after castration instead of the usual three weeks. It is to be noted (Table 2) that this animal had the highest 17-ketosteroid excretion of any castrate.

Experimental cryptorchidism. Three normal rabbits were made cryptorchid by retracting their testes into the abdominal cavity and

TABLE 2. COMPARISON OF THE AVERAGE EXCRETIONS OF TOTAL 17-KETOSTEROIDS AND URINE VOLUMES FOR INDIVIDUAL ANIMALS IN NORMAL AND CASTRATE CONDITIONS

Animal	Normal excretions			Castrate excretion			Per cent. decrease
	No. of urines	17-KS mg./48 hrs.	Urine ml./48 hrs.	No. of urines	17-KS mg./48 hrs.	Urine ml./48 hrs.	
B	9	2.27 ± 0.19	402	4	1.79 ± 0.23	378	21.1
H	3	2.56 ± 0.27	852	3	1.64 ± 0.48	846	39.9
I	6	2.35 ± 0.08	318	3	1.45 ± 0.17	338	38.1
J	4	2.50 ± 0.05	459	3	0.96 ± 0.09	418	62.0
K	4	2.76 ± 0.34	467	3	1.52 ± 0.33	536	44.8
L	4	2.01 ± 0.19	270	3	1.37 ± 0.01	225	31.8
M	3	1.88 ± 0.38	422	3	1.32 ± 0.50	356	29.7
O	3	1.91 ± 0.23	283	4	0.82 ± 0.50	392	56.8
P	—	2.31 ± 0.10*	—	4	1.29 ± 0.35	373	44.1
Q	—	2.31 ± 0.10*	—	4	1.28 ± 0.19	307	44.5

* Excretion level of colony.

ligating them to the abdominal wall with minimal disturbance to circulation. As an added precaution the gubernaculum was ligated and cut. A total of seventeen 48-hour urines were assayed. Animals P and Q were identical with the rest of the colony and a normal excretion level was not determined separately for these two animals.

In order to establish a definitive condition of cryptorchidism a period of four weeks elapsed before collection and assay of the urines. The level of 17-ketosteroid excretion was that of castrates (Table 1). To determine subsequent effects of prolonged experimental cryptorchidism a period of eight weeks was allowed to elapse after the previous urine collections and a second series of collections were

TABLE 3. COMPARATIVE EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM AND SUBSEQUENT CRYPTORCHIDECTOMY REFLECTED IN THE AVERAGE TOTAL 17-KETOSTEROIDS EXCRETION (MG./48 HRS.) OF INDIVIDUAL ANIMALS. THE NUMBER OF 48-HOUR COLLECTIONS ASSAYED ARE IN PARENTHESES

Animal	Normal excretion	Cryptorchid excretion	Castrate excretion
M	1.88 ± 0.38 (3)	1.28 ± 0.12 (6)	1.32 ± 0.50 (3)
P	2.31 ± 0.10 (—)	1.54 ± 0.36 (6)	1.29 ± 0.35 (4)
Q	2.31 ± 0.10 (—)	1.40 ± 0.07 (5)	1.28 ± 0.19 (4)

made. The results confirmed the first assay series and established the 17-ketosteroid excretion at the depressed level of castrates.

The cryptorchid rabbits were converted subsequently to castrates by cryptorchidectomy. The gonads were removed and examined microscopically. Sections showed the tubule degeneration characteristic of cryptorchid testes while interstitial cells appeared unaffected. Three weeks after castration eleven 48-hour urine collections were made and assayed. There was no significant change in 17-ketosteroid excretion from the cryptorchid condition (Table 3).

Testosterone pellets. The effectiveness of an androgenic precursor upon the 17-ketosteroid excretion was studied in two castrated animals. Rod-shaped pellets of crystalline testosterone (Oreton F, Schering) weighing 25.0 mg. were used. A single pellet was inserted subcutaneously into the medial left thigh region of each animal. Two days following pellet implantation urines were collected continuously for 14 days. At the end of this period the pellets were removed, dried and weighed. The pellet removed from animal H indicated an absorption of 6.8 mg., while the pellet recovered from rabbit K had decreased 7.2 mg. in 16 days.

Table 4 describes the conditions of the experiment and the 17-ketosteroid excretion for the last 14 of the 16 day period. The values indicate an immediate effective increase of 17-ketosteroid excretion within two days of implantation and than a gradual increase for the next 10 days. The greatest increase during the observed period occurred 15 and 16 days after implantation of the pellets.

TABLE 4. EFFECT OF IMPLANTED TESTOSTERONES PELLETS (25.0 MG.) UPON THE CONTINUOUS 17-KETOSTEROIDS EXCRETION (MG./48 HRS.) OF CASTRATED MALE RABBITS. THE NUMBER OF URINES ASSAYED ARE IN PARENTHESES

Animal:	H	K
Normal excretion:	2.56 ± 0.27 (3)	2.76 ± 0.34 (4)
Castrate excretion:	1.64 ± 0.48 (3)	1.52 ± 0.33 (3)
Pellet absorption: (By 16th day)	6.8 mg.	7.2 mg.
Collection period	17-KS excreted	17-KS excreted
Days 1-2	—	—
3-4	2.25	2.63
5-6	1.71	2.75
7-8	2.34	2.41
9-10	3.14	3.05
11-12	3.05	2.48
13-14	3.18	3.64
15-16	5.41	6.00

Injection of pituitary material. The effect of anterior pituitary extracts upon the 17-ketosteroid excretion of low-normal animals was investigated in three rabbits. The pituitary extract used was Polyansyn (Armour) which is a polyvalent extract of pituitary prepared after the method of J. B. Collip. Each animal (R, S and T) received subcutaneously 6 cc. of Polyansyn in divided doses over a six day period. Urine collections were made for 14 days following the first injection. Table 5 lists the excretion of 17-ketosteroids prior treatment compared with excretion during treatment. With the possible exception of animal S there was no significant change in 17-ketosteroid excretion.

While the normal values of these animals were exceptionally low there were no discernible pathologies which would invalidate the assay values obtained from them.

DISCUSSION

Urine volumes did not vary significantly for any experimental condition. An occasional individual rabbit consistently excreted unusual volumes of urine. For example, animal N had an average excretion of 1,080 ml./48 hours, whereas the group average was 357 ml./48 hours. Of all animal groups the normal animals yielded the

TABLE 5. RESULT OF INJECTING LOW-NORMAL ANIMALS WITH PITUITARY EXTRACTS UPON THE TOTAL 17-KETOSTEROIDS EXCRETION (MG./48 HRS.). THE NUMBER OF URINES ASSAYED ARE IN PARENTHESES

Animal:	R	S	T
Excretion prior treatment:	1.78 ± 0.05 (4)	1.28 ± 0.15 (4)	1.93 ± 0.12 (3)
Excretion during treatment:	1.60 ± 0.24 (4)	1.60 ± 0.22 (4)	1.97 ± 0.27 (4)

greatest variation in urine volume of any group and varied between 154 to 1,465 ml./48 hours.

In accordance with the evidence obtained gonadectomy depresses the 17-ketosteroid excretion of male rabbits (Tables 1 and 2). Average values obtained before and after castration indicated the contribution of the testes in rabbits is greater than with man. The replacement value of testosterone pellets corroborates further the importance of testicular secretions to urinary 17-ketosteroids. Subcutaneous testosterone pellets produced a four-fold increase in 17-ketosteroid excretion within 16 days of implantation. Further increases may have been observed with more prolonged study of the urine excretions. The relatively high excretion occurring seven months after castration of animal B is suggestive of a compensatory mechanism developed with time after castration. Such adrenal compensation for gonadal secretions has been postulated for high levels of the female climacteric (Hamblen et al, 1941), and for the increase in 17-ketosteroids excreted by castrated patients with prostatic cancer (Scott and Vermeulen, 1942). The changing proportions of constituent 17-ketosteroids (e.g. more dehydroisoandrosterone) in human castrate urine also suggests adrenal compensation in the absence of the testes (Callow and Callow, 1940).

Considerably more difficult to interpret is the depressed excretion of cryptorchid animals. Evidence obtained in the present study would indicate as early as thirty days following experimental cryptorchidism in rabbits in the 17-ketosteroid excretion is reduced to that of a castrate within experimental error of the technique. Assays of urines collected sixty days after surgery indicate no further decrease in 17-ketosteroid excretion. No further decrease with subsequent cryptorchidectomy adds corroborative evidence of the castrate-like excretion of 17-ketosteroids during experimental cryptorchidism. There is evidence indicating animals made cryptorchid do not continue to secrete the normal level of androgens (Korenchevsky, 1930; Nelson, 1934; Hanes and Hooker, 1937). The depressed 17-ketosteroid excretion suggests that normal 17-ketosteroid level of the cryptorchid rabbit is only transitory at best. An alternative possibility suggests that the high internal body temperature induces an aberrant testicular hormone metabolism, still testoid, which might not be excreted as a 17-ketosteroid. Such aberrant metabolism induced by thermal change is postulated for androgenic activity of the ovary transplanted to the external ear of male mice (Hill, 1941).

For a study of pituitary stimulation it was thought advantageous to use animals which yielded very low normal 17-ketosteroid excretions since such animals might be pituitary deficient to a mild degree. It is also entirely possible that such animals are refractory to pituitary stimulation. Fraser et al. (1941) obtained increased excretion in hypogonadal patients showing primary pituitary deficiency with adminis-

tration of chorionic gonadotropin, while male castrates and eunuchoids with positive F.S.H. (gonadal deficient) showed no change. Possibly the dosages used were not sufficient to stimulate activity of the target organs since massive doses of purified adrenocorticotrophic hormone have been found to increase 17-ketosteroid excretion in man (Mason et al., 1948; Forsham et al, 1948).

SUMMARY

Partially purified neutral ketonic fractions of rabbit urine were assayed for total 17-ketosteroid content by an anhydrous Zimmerman reaction. In the male rabbit castration produces an average decrease of 41% in the total urinary excretion of 17-ketosteroids. Experimental cryptorchidism depresses the normal 17-ketosteroid excretion to castrate level by thirty days. Subsequent cryptorchidectomy does not further decrease the 17-ketosteroid level. With moderate dosage, a polyvalent pituitary extract (Polyansyn, Armour) does not raise the 17-ketosteroid excretion of low-normal animals. Implantation of testosterone pellets into castrates causes a four-fold increase in 17-ketosteroid excretion sixteen days after implantation.

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PRESIDENTIAL ADDRESS AT THIRTIETH ANNUAL
MEETING OF THE ASSOCIATION FOR THE
STUDY OF INTERNAL SECRETIONS

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Members of the Association and their guests, ladies and gentlemen:

THE CONSTITUTION of the Association contains no provision that the President should address the members at their annual meeting, nor for that matter does it compel the membership to listen to him, yet the custom of our society has always been that this occasion is one at which the President is privileged to speak on matters pertaining to our common interest in endocrinology. In recent years your President's responsibility has been lightened by the fact that the address is usually delivered after the annual dinner and after a preliminary gathering which is nicely calculated to bring his audience into its most receptive mood. This adroit timing of the address not only enables the listeners to endure what is to follow but is in sharp contrast to the custom of another eminent medical society, which also once honored me with their presidency. Their barbarous custom was and is to compel the incumbent to address his unfortunate colleagues immediately after an early breakfast. For all concerned it was and is a chilling and disheartening experience. Indeed its effects are such as to remind me of the classical reply of the eminent British scientist who was invited to speak at a scientific meeting in this country. On his arrival he was informed by the secretary of the society concerned that his paper was scheduled for 7 A.M. the next day. Without a pause he replied "Young man I never stay up as late as that."

However, before I begin to speak on a more serious note I want to take this opportunity to convey to the members of the Association my sincere appreciation of the privilege that it has been to serve as your President during the past year. The presidency of this association is not only an honor but a responsibility. The honor is one I shall always cherish; the responsibility could not have been met had it not been for the cooperation of your Council and above all the hard work and tireless enthusiasm of the Secretary-Treasurer. The Association owes a deep debt of gratitude to Dr. Henry Turner and one that I consider it a privilege to acknowledge this evening. It is also

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proper at this time to extend the appreciation and thanks of the Society to Dr. Willard Thompson and the other members of the local committee for the work they have done to make this meeting such a pleasant occasion for us all.

The question as to what I should devote the main body of my address this evening has given me no little concern. Judging from past years there were many possibilities ranging all the way from an interesting travelogue by one past president to rather detailed scientific papers by others. Since my peregrinations are of little interest and since we have a very full program of papers this year, it seemed more appropriate that I should spend a little time to discuss with you the past, present and, so far as one man can see, the future developments of endocrinology.

It is now thirty-one years since the first volume of ENDOCRINOLOGY was published and it is a salutary experience to re-read the papers presented in it, particularly if this is followed by a perusal of the volumes which were published in 1947. In volume I there appeared eighteen original articles, in the two volumes for 1947 a total of 126 original papers, to say nothing of those appearing in the *Journal of Clinical Endocrinology*. Yet these figures give a most inadequate picture of the changing character of this science. Among the sixteen papers in the first volume we note one by Brailsford Robertson on "Tethelin," the growth controlling constituent of the anterior pituitary, a principle stated to be active by mouth and stable at 80° C., a paper that drew cautious and guarded comment from Dr. Goetsch, for in those days the discussion of papers given at the Association meeting was printed in the journal. We also find in this first number the classical paper by Kendall on the isolation of thyroxine and its effects on human cretinism and myxoedema. But aside from these articles which have a flavor of modern experimental endocrinology, the remainder are essentially of a descriptive character. By this I mean they report the over-all effects of certain types of endocrine hypo- or hyperfunction in man and in reality represent the emergence of endocrinology from the world of giants, dwarfs, bearded ladies and the other oddities produced by extreme degrees of endocrine dysfunction with which it had for so long been identified.

I choose the term "emergence of endocrinology" with deliberation since it is my belief that we have now reached the stage when the functions of the endocrine glands are well enough understood to appreciate that they constitute a bodily system of which it is more important to know the basic knowledge of their mode of action and the minor aberrations of such function than it is to consider them only when they present themselves in such usually rare forms as Addison's disease, Cushing's syndrome, etc.

The emergence of endocrinology from the age of curiosities to a full fledged basic and clinical science has depended on three main

pathways of research. The first of these is the identification and isolation of the hormones themselves. In 1917 two hormones were clearly recognized and only one chemically identified. They were of course epinephrine and thyroxine; to these may be added the recognition of the activity of posterior lobe extracts. Today there has been isolated practically every known hormone, the majority in pure form. This phase of endocrinology may well be said to have been begun by the isolation of insulin in 1921, followed by the synthesis of thyroxine in 1926, the isolation of estrone in 1929, of testosterone and androsterone in 1935, the identification of the active adrenal cortical hormones as steroids in the early 1930's. The recognition that a protein, insulin, could also be a hormone led to a sustained attack on the other protein hormones, notably those of the anterior pituitary. Such success has now been achieved that the adrenotrophic, lactogenic, growth and gonadotrophic hormones have all been obtained in substantially pure form leaving only the thyrotrophic and possible other at present unidentified pituitary factors to the wiles of the protein chemist. Among the other protein hormones work still remains to be done on that of the parathyroid and certain gastrointestinal hormones.

These achievements carried out in several countries and by many different investigators have not only presented the physician with a remarkable armament of therapeutic agents but have led the way for the dissection of function of the endocrine system that is so essential for our understanding of its extraordinary effects on bodily processes. Their potency has also compelled a degree of caution in their use which is wholly desirable for medicine, for while our colleagues of thirty years ago may have fed anterior pituitary extracts with reckless abandon, no physician should, after what we have heard this afternoon, inject adrenotrophic hormone without careful consideration of its potent diabetogenic qualities. Indeed, I like to think that one of the important contributions of our meetings is to emphasize these qualities of present day endocrine preparations as much as to record our success in their isolation.

Therefore, so far as the isolation of the active principles of the known endocrine glands is concerned, it may be said to be substantially complete. The chemical structure of all except the protein hormones is rather exactly known; synthetic methods have been developed for epinephrine, thyroxine and many steroid hormones and are being continually increased. The contributions of the chemist to endocrinology in the last thirty years are perhaps the most outstanding event of this period.

The second line of endeavor that has enormously broadened our concept of endocrinology has been the parallel advances in our understanding of the intermediary metabolism of cells. This has included not only an analysis of the chemical steps involved but also the isolation and identification of the enzymes themselves. Not only have

major factor. There is also some evidence in the case of the adrenotrophic hormone that the release of epinephrine by activation of the autonomic nervous system is a preliminary event. Yet we do not know the role, if any, of nerve elements that may reach these cells nor do we know how the selective discharge of pituitary hormones is affected. It appears unlikely at first sight that the release of F.S.H. and L. H. or of growth hormone and adrenotrophic hormone would occur simultaneously. Rather it would appear that the time sequence of discharge is adjusted in some manner to the circumstances prevailing in the body. Furthermore, we do not know whether in the period of rapid growth if the presumed excess of growth hormone in the blood is due to an increased secretion by the gland or a lowering of the rate of release of adrenotrophic hormone while maintaining a constant output of growth hormone. There are dozens of such questions relating to the function of the thyroid, gonads and adrenal cortex that require information about the regulation of anterior lobe secretion for their solution.

I have spoken several times of the importance of the blood levels of certain hormones as a determinant of endocrine activity. We are greatly handicapped in clinical endocrinology by our inability to find methods that will enable us to analyze or assay for such blood concentrations. Dependence on urine levels for the assessment of the secretory activity of an organ places us in the same position as were the clinicians of thirty years ago who had only urinary glucose measurements to guide their diagnosis and treatment of diabetes mellitus. We can anticipate an equally great increase in our ability to diagnose and treat endocrine disorders were we in a position to follow the blood levels of the various hormones.

Mention of diabetes mellitus calls to mind that perhaps with the exception of gonadal disturbances in the female that this is the most prevalent endocrine disorder encountered in man. Possibly two million people in this country suffer from an absolute or relative insufficiency of insulin secretion. While the isolation of insulin has enabled us to control the disease once it has presented itself, we are still largely in the dark as to the reasons for its onset. In no endocrine disorder is the collaboration of the student of intermediary metabolism and the endocrinologist more essential for the solution of the etiology of this disease. Much the same can be said for human hyperthyroidism. The advances in our understanding of thyroid physiology have been of a spectacular character and have led to the development of agents that can control hyperthyroidism by blocking out enzyme reactions essential for the formation of the hormone, yet here again little is known of the chain of events that lead to the clinical picture that ultimately presents itself for our relief.

A whole evening could be devoted to the shortcomings in our knowledge of gonadal dysfunction, particularly in the female. Replacement therapy at times brings spectacular results; in many others

it is quite incapable of re-initiating the normal sequence of events. Here perhaps as in no other situation is the coordinated and integrated activity of several endocrine glands an essential feature of normal physiological activity. A measure of our distance from the goal is to be seen in our failure to re-establish normal reproductive function in hypophysectomized animals.

I have always been interested in the comparatively small amount of attention that has been paid to the endocrinology and intermediary metabolism of pregnancy. Here is a physiological event quite evidently associated with widespread adjustments in the endocrine system and a sustained and additional burden on the processes of metabolism and yet I think it is fair to say that we know more of some rare endocrine disorders than we do of these events. Of equal interest, at least to me is the participation of certain hormones in the production of lactation. The mammary gland forms in its cells a unique protein, casein, a unique carbohydrate, lactose and fats of a type found only in milk. All these synthetic processes depend upon an adequate supply of the lactogenic hormone of the anterior pituitary and we would dearly like to know not only more about these unique metabolic reactions but also the relation of this hormone to them.

The present age finds the scientists of this country engaged in the most broad attack on the problems of malignancy that has yet been undertaken. Among the many fields that are being given particular attention is the relation of the steroid hormones, both to the genesis and treatment of certain types of malignancy. Promising results have been obtained by applying our knowledge of endocrinology to the treatment of malignancies arising in tissues that are known to be under hormonal control such as the breast and prostate. Others are investigating the hypothesis that aberrations of normal steroid metabolism may be the etiological basis of malignancy—a hypothesis that will not only require long and painstaking experimentation but most certainly necessitates an understanding of the effects of age, nutritive factors and the mechanism of normal hormone metabolism before decisive answers can be obtained.

I could continue to remind you for some time of the scantiness of our knowledge both in experimental and clinical endocrinology and one might despair of our ability to answer such questions were it not that contemplation of the advances in the thirty years since the establishment of the Association revives our hopes for the future. Thirty years ago endocrinology was struggling to be regarded as a respectable field of research; today this Association and its journals represent only a small part of the individuals who are working in this field, not perhaps calling themselves endocrinologists but appreciative of the fact that the capacity of the organism to regulate its function to its needs by means of hormones is vital to our understanding of normal and abnormal physiology.

In conclusion, I would like to say a few words to those physicians

whose problems lie in clinical endocrinology as it presents itself in their daily practice. Many have expressed to me their sense of frustration that the vast volume of experimental work appears at first sight to bear so little relation to human disease. I can only reply that these basic studies are essential, contradictory as they may often appear, yet the physicians should not forget that the contributions of the clinician have been and may be just as important as those from the laboratory. The names of Addison, Graves, Pierre Marie should remind us that careful observation and controlled studies in man are just as pertinent today as they were in their time. I regard it a privilege to have been the President of a society in which there is such a happy mingling of the laboratory worker and the clinician and I hope that the Association both in its membership and in its program will continue to reflect our joint interest in the problems of the function of the endocrine organs in health and disease.

MECHANISM OF INACTIVATION OF α -ESTRADIOL BY RAT LIVER "IN VITRO"¹

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IT IS WELL known that the liver is the most important site of estrogen inactivation in different species. The early work of Zondek (1934) has shown that 95 per cent or more of the biological activity disappears when estrone is administered to man or rats or is incubated with rat liver "brei." Heller (1940) studied the inactivation of estrone, estradiol and estriol by slices of various tissues and the influence of several inhibitors upon this phenomenon. Engel and Rosenberg (1945) have observed that aqueous liver extracts, obtained at different pH, inactivate estrone in prolonged incubation. More recently Levy (1947), working with a liver extract prepared by a modification of Zondek's (1934) technique, has been able to study several properties of the inactivating system. The present study was undertaken for the purpose of securing additional information regarding the nature of the estrogen-inactivating mechanism.

METHODS AND TECHNIQUES

Male Wistar descendants, weighing from 150 to 300 gm. were used throughout. Different types of liver preparations were employed. Rats were killed by a blow on the head and section of the neck with scissors. The liver was immediately removed and was either sliced according to the well known Warburg technique or subjected to the following procedures:

Three other preparations were used, i.e., frozen powder, homogenate and acetone powder.

(1) The powder was prepared by freezing liver with a mixture of dry ice and ethyl alcohol and pulverizing the hardened tissue in a mortar. A 10 per cent suspension of the powder in Krebs (1933) solution was used for the experiments.

(2) A 10 per cent homogenate was prepared. The required amount of liver was dropped in an ice-cold suspending medium, previously placed in the glass container of a Waring Blendor. The glass container was kept cold by packing with crushed ice and the tissue was homogenized for 3 minutes in a cold room at approximately 5° C.

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(3) The dry acetone powder was prepared according to the technique of Kaplan and Lipmann. The livers were rapidly removed and placed in an ice-packed dish. While cold, they were finely minced with scissors. The mince was transferred to the glass container of a Waring Blendor with 20 volumes of acetone, kept cold by packing with ice, and homogenized for two minutes in a cold room ($5^{\circ}\text{C}.$). The homogenized material was allowed to settle for a few minutes, and filtered through a Buchner funnel of such size that the solid would make a layer about 1 cm. deep. It was not allowed to dry while filtering, and was washed twice with cold acetone and twice with peroxide-free, dry ether. The washings were done at room temperature. The material was sucked dry and thoroughly dried in a vacuum desiccator over P_2O_5 for about an hour. The dry powder was passed through a 20 mesh sieve to remove connective tissue and stored in a stoppered bottle placed in a deep freeze chamber. When used, it was suspended in a convenient medium.

Three types of medium were used: (1) Krebs (1933) solution, (2) Fuhrman and Crismon (1944) "intracellular medium," and (3) 0.1 M sodium phosphate buffer, all at pH 7.4, buffered with phosphate.

The preparations, 100 mg. of slices, 10 per cent powder or homogenate or the equivalent amount of acetone powder, were incubated for two hours with constant shaking at $37.5^{\circ}\text{C}.$ in 50 cc. Erlenmeyer vessels containing a total volume of 5 cc. with 1 or 2 $\mu\text{g}.$ of α -estradiol per cc. A control, in which the α -estradiol was added at the end of the incubation period, was run in the same manner. An adequate amount of a solution of α -estradiol in 50 per cent ethyl alcohol containing 100 $\mu\text{g}.$ per cc. was added to each vessel to attain the required concentration. After incubation, approximately 25 cc. of acetone were added to each vessel, the content of two vessels was pooled, and the biological activity determined, in castrated mice, by a modification of the Allen-Doisy technique (1923).

The error of assay was ± 25 per cent when using 10 mice per dose and ± 16 per cent for 20 mice per dose. For this reason only differences greater than 25 per cent were considered significant. The biological activity recovered after incubation was expressed in terms of the equivalent amount of α -estradiol.

In varying the conditions of incubation we have found that the several preparations used showed the same activity whether the gas phase was oxygen or air. For this reason, after the initial experiments, the aerobic incubations were carried out in air.

The boiled liver extracts used in some of the experiments were prepared by boiling liver mince, homogenate or liver slices in Krebs solution for 5 minutes, centrifuging and using the supernatant. In experiments with boiled mince and homogenate the amount of boiled extract equivalent to 0.5 gm. of liver was used in each vessel; with boiled slices extract the amount corresponded to 0.15 gm. of liver.

In the starvation experiments the rats were starved for 4 days.

The thiouracil treated rats received thiouracil in the drinking water (containing 0.5 mg. thiouracil per cc.) over a period of 60 days.

RESULTS AND COMMENT

Activity of Different Liver Preparations. The behavior of the liver preparations is shown in Table 1. Certain conclusions seem justified on the basis of these findings.

LIVER INACTIVATION OF α -ESTRADIOLTABLE 1. INACTIVATION OF α -ESTRADIOL BY DIFFERENT MALE RAT LIVER PREPARATIONS

Preparation	Medium	No. of exper.	Initial α -estradiol	Recovery*	Inactivation %
Slices (control)	Krebs	4	7	10	0
Slices	Krebs	10	10	7	>70-100 (>93)
Slices	Krebs	10	10	10	>85-98 (>96)
Boiled slices	Intracellular	10	20	<3-0 (<0.7)	96; 97 (96.5)
Crushed frozen liver	Krebs	2	20	<3-0.4 (<0.8)	0
Homogenate (control)	Krebs	2	10	0.8; 0.6 (0.7)	70-90 (82)
Homogenate	Krebs	2	20	10	0; 20 (10)
Homogenate (control)	Krebs	2	10	6-2 (3.7)	20-70 (42)
Homogenate	Krebs	13	10	10; 8 (9)	0; 10 (5)
Homogenate (control)	Krebs	12	10	8-3 (5.8)	20-70 (55.5)
Homogenate	Intracellular	12	20	20; 18 (19)	10; 20 (15)
Homogenate (control)	Intracellular	12	20	16-6 (8.9)	70; 70 (70)
Homogenate	0.1 M phosphate buffer	8	20	18; 16 (17)	0-36 (23)
Homogenate supernatant	0.1 M phosphate buffer	9	10	6; 6 (6)	10- >70 (46)
Homogenate sediment	0.1 M phosphate buffer	2	20	10.3-6.4 (7.7)	<50; 70 (60)
Acetone powder (control)	0.1 M phosphate buffer	2	20	18- <6 (10.7)	40; 50 (45)
Acetone powder	Krebs	2	20	>10; 6 (8)	0; 12 (6)
Acetone powder	0.1 M phosphate buffer	2	10	12; 10 (11)	50-68 (57)
Acetone powder	Krebs	4	20	11; 8.8 (9.9)	50; 70 (60)
	0.1 M phosphate buffer	2	20	10-6.4 (8.6)	
	Krebs	2	20	10; 6 (8)	

* Determined by bioassay and expressed as α -estradiol.

Note: Figures in parentheses indicate the average. In controls estradiol was added at end of incubation.

Fresh liver slices completely inactivated α -estradiol, while boiled slices had no inactivating effect.

The inactivation obtained with frozen pulverized liver, with the 10 per cent homogenate and with the acetone powder, was of the same order of magnitude, ranging from 20 to 90 per cent. No change in the behavior of the preparation was observed if the Fuhrman and Crismon (1944) "intracellular medium" was substituted for the Krebs (1933) solution or if 0.1 M sodium phosphate buffer pH 7.4 was used; addition of glucose had no effect.

The activity of the homogenate did not change appreciably after 24 hours dialysis at 5° C. against distilled water or against 0.1 M phosphate buffer pH 7.4.

The supernatant of the homogenate, obtained by centrifugation at 3000 r.p.m., showed the same activity as the original preparation. The sediment, brought to the initial volume, was less active.

Effect of Inhibitors. The action of several enzyme inhibitors on the activity of liver slices was tested with the purpose of gaining information regarding the properties of the inactivating system or systems. The inactivation was completely inhibited when incubation was carried out in a nitrogen gas phase.

Several inhibitors were used and, as indicated in Table 2, only cyanide (70 per cent inactivation), azide (90 per cent inactivation) and capryl alcohol (30 per cent inactivation) exerted any appreciable inhibitory effect.

No inhibition of inactivation was observed in presence of malonate, monoiodoacetate, iodoacetamide, fluoride and thiourea.

Inactivation as a Dehydrogenation. Several experiments indicated that the inactivation of α -estradiol is at least partially accomplished by dehydrogenation.

liver. The addition of 0.001 to 0.005 M citrate to our type of homogenate did not increase the inactivation of α -estradiol (Table 4).

TABLE 4. INFLUENCE OF OTHER FACTORS IN α -ESTRADIOL INACTIVATION BY MALE RAT LIVER

Preparation	Effect of	No. of exp.	Initial α -estradiol	Recovery*	Inactivation
Homogenate (control)	—	3	7 10	10.5-8.2 (9.6)	0-18 (6)
Homogenate	—	6	20	18-7.6 (11.4)	10-62 (43)
Homogenate	0.001 M citrate	3	20	11.4-6.4 (9)	43-68 (55)
Homogenate	0.002 M citrate	2	20	12.8; 9.5 (11.2)	36; 52 (44)
Homogenate	0.005 M citrate	5	20	12.3-10.8 (11.5)	38-46 (42)
Slices	Thiouracil treated rats	4	10	(<0.6)	(>94)
Homogenate (control)	Thiouracil treated rats	1	10	10	0
Homogenate	Thiouracil treated rats	4	20	8.2-6 (7.2)	59-70 (64)
Slices (control)	Starvation	1	10	12	0
Slices	Starvation	4	10	2.4- <0.6 (<1.3)	76- >94 (>87)
Slices (control)	0.43 mM phenol	1	20	20	0
Slices (control)	—	1	10	10	0
Slices	—	1	20	0.6	97
Slices	0.43 mM phenol	2	20	0.75; 0.3 (0.5)	98; 98.5 (97.5)

* Expressed as α -estradiol, determined by bioassay.

Note: Figures in the parentheses indicate the average.

With the idea that phenol might inhibit the inactivation, acting as a competitive substrate with the phenolic part of the α -estradiol molecule, we added 200 μ g. of phenol per vessel (a concentration 10 times higher than that of the α -estradiol used). No effect on the inactivation was observed.

The livers of thiouracil-treated rats inactivated α -estradiol to the same extent as those of the normal rats.

A slight decrease in activity was noticed in livers of rats starved for 4 days; further experiments will be necessary to confirm this observation.

CONCLUSIONS

As shown by previous workers, liver slices (Heller, 1940) and "brei" (Zondek, 1934) inactivate estrogens. The process is enzymatic, inasmuch as boiled liver slices show no such activity. The transformation seems to be oxidative in nature as it does not occur in a nitrogen gas phase. That a dehydrogenating mechanism is at least partially involved in the process of inactivation is indicated by the observation that addition of methylene blue reestablishes in part the activity of slices incubated anaerobically. We believe that methylene blue acts as a hydrogen acceptor and that, under these conditions, estradiol may possibly be converted to estrone.

The dehydrogenation is in part linked to DPN. This is indicated by the increase in activity of the homogenate after addition of DPN and by the augmenting effect of nicotinamide. The latter probably produces this effect by decreasing the breakdown of DPN, as it has been shown by Mann and Quastel (1941) and Handler and Klein

(1942) to inhibit the breakdown of this coenzyme by different tissue preparations. This property may explain the great difference observed between the activity of slices and the other types of preparation, i.e., frozen powder, homogenate, acetone powder. Hydrolysis of the products of incubation produces no increase in recoverable activity. This suggests that conjugation has not occurred, which is in agreement with the findings of Heller (1940). However, in view of the error inherent in the assay procedure, this possibility cannot be entirely ruled out inasmuch as Zondek (1934) by hydrolysis of the carcass of rats, was able to recover 20 per cent of the estrogenic activity of the estrone administered during the preceding 24 hours.

The rather slight inhibition of inactivation produced by cyanide and azide, contradicting the observations of Heller (1940) and Levy (1947), indicates that the system cytochrome-cytochrome oxidase does not participate to a large extent in the process of inactivation under the conditions of our experiments.

The effect of the various inhibitors studied indicates that heavy metal and calcium-containing enzymes, -SH enzymes and succinic acid dehydrogenase are not involved in the hepatic inactivation of α -estradiol in vitro.

SUMMARY

The inactivation of α -estradiol by different types of male rat liver preparation has been studied. A 2-hour incubation was carried out in 5 cc. of medium (Krebs solution, Fuhrman and Crismon "intracellular" or 0.1 M sodium phosphate buffer, all buffered with phosphate at pH 7.4) at 37.5°C. Usually 1 or 2 μ g. of α -estradiol per cc. was used. The amount of biologically active material present at the end was determined by bioassay in spayed mice.

Complete inactivation by liver slices was observed in oxygen or air. No inactivation took place in nitrogen or with boiled slices.

The 10 per cent frozen liver powder, homogenate or the equivalent amount of acetone powder produced 20 to 90 per cent inactivation. Neither the type of medium used nor the presence of glucose influenced these results.

The activity of the homogenate was not changed appreciably by 24 hours dialysis. Boiled liver extract prepared from mince or homogenate did not increase the activity of the homogenate or dialysate, but the boiled extract obtained from fresh liver slices produced a detectable increase.

Centrifugation of the homogenate at 3000 r.p.m. gave a supernatant of the same activity, but the sediment brought to the initial volume was less active.

A slight degree of inhibition of inactivation was observed in the presence of cyanide and azide (70 to 90 per cent inactivation). Only 30 per cent inactivation occurred in the presence of capryl alcohol. No

inhibition was observed in the presence of malonate, monoiodoacetate, iodoacetamide, fluoride and thiourea.

The addition of methylene blue to slices incubated in nitrogen brought about 60 per cent inactivation.

Nicotinamide and DPN added to the homogenate increased the inactivation by approximately 50 per cent; no further increase was observed when cytochrome C was added. Homogenate prepared with nicotinamide showed greater activity than that obtained in its absence.

Citrate, phenol and the feeding of thiouracil had no effect on estradiol inactivation. A slight decrease was noticed when the rats were starved for 4 days.

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THE ANTITHYROID EFFECT OF CERTAIN FOODS IN MAN AS DETERMINED WITH RADIOACTIVE IODINE¹

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ALTHOUGH iodine deficiency is well established as an etiologic factor in endemic goiter, it sometimes appears that other agents may be responsible. It is evident that the sporadic goiter which occurs in a "goiter-free" area where there is a relatively high concentration of iodine in the soil and water cannot be satisfactorily explained on an iodine deficiency basis.

Before the discovery of iodine, investigators had advanced many hypotheses as to the cause of goiter, but with the general acceptance of the "iodine-lack" theory in the second and third decades of this century most of the earlier ideas were discarded. In view of the inability of this "deficiency" theory to explain many instances of this disease, however, other causes have been sought.

For many centuries the thought has existed that dietary habits might be responsible for endemic goiter. The Tyroleans, for instance, believed that their "swelled necks" were caused by eating a type of Alpine chestnut (Barton, 1800). Around the turn of the last century several investigators claimed to have shown a goitrogenic effect of certain foods in laboratory animals, but it was not until 1928 that interest was truly stimulated in the possibility that food might be a cause of this disease. In that year, Chesney, Clawson, and Webster (1928) reported that a colony of rabbits they were maintaining for work in experimental syphilis developed large goiters while on a daily ration of cabbage. After considering other factors which might have been responsible, these workers concluded that the cabbage per se was the etiologic agent. In other words, there was a positive effect exerted by this vegetable which caused thyroid enlargement in spite of an adequate iodine intake.

This experiment has been confirmed several times in the twenty years that have followed. Since the original demonstration of activity in cabbage, a variety of foods, including chard, rape, turnip (*Hercus*

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and Purves, 1936), soybeans (Sharpless, 1938, McCarrison, 1934), and peanuts (McCarrison, 1937), has been claimed to cause goiter in both laboratory animals and livestock.

Since cabbage is a member of the family Cruciferae, it is not strange that the majority of the investigations has been carried out on this group. This family is generally thought to possess stronger goitrogenic potency than other plants, with the genus *Brassica* (containing cabbage) seemingly its most active subgroup. Hercus and Purves (1936) have also shown that *Brassica* seeds produce considerable thyroid hyperplasia in the rat. Most of the search for goitrogenous food has been confined to plant derivatives, but some animal products have also been incriminated, among them fats and liver.

The active agent responsible for the goitrogenic effect of these foodstuffs has not been identified with certainty as yet. When it seemed apparent that most of the Brassicace caused thyroid hyperplasia, it was postulated by Marine et al. (1931) that the mustard oils (isothiocyanates), the most characteristic compounds of this genus, were the ingredients involved. These, however, proved inactive when tested in the rat. After trying several other compounds contained in these plants, Marine's group found that the cyanides were quite goitrogenic, acetonitrile being the most active.

Many workers have been unable to confirm this action of cyanide, however. With the discovery of the goitrogenic properties of thiourea and related compounds, it was postulated that the active substance in foods might well be one of this group. Since it was known that the mustard oils could be converted by ammonia to thioureas, allylthiourea was suggested by Kennedy as a possibility. As yet, however, attempts at isolation of an active goitrogenic compound from foodstuffs have failed.

Thiocyanate has also been shown to cause thyroid enlargement (Barker, 1936). It has now been well demonstrated that the action of this substance is to prevent the selective concentration of the iodide ion in the thyroid and to lead to the development of goiter if the iodine intake is sufficiently low. The organic binding of iodine seems to be unaffected (Wolf et al., 1946, VanderLaan and VanderLaan, 1947). Thiocyanate, therefore, must be added to the list of possibilities.

A serious drawback in attempting to interpret the results of these animal experiments in their relation to the pathogenesis of goiter in man is the marked species difference which exists in the response to antithyroid agents. It would be instructive to assay various foodstuffs directly on human subjects; but until quite recently this has been impractical. However, the determination of antithyroid activity by observing the inhibition of the radioactive iodine uptake (Stanley and Astwood, 1947) was found to provide a rapid and convenient method for assaying the effect of various foods in man. In this paper the results of testing a number of the more common foods by this method are reported.

METHODS

The subjects were technicians, medical students, and physicians. They were asked to omit breakfast and at 9 A.M. they were given a tracer dose of 100 microcuries of radioactive I^{131} in 10 cc. of physiological saline. This was followed by 10 cc. of tap water to wash the remaining iodine into the stomach from the flask, mouth, and pharynx. The uptake of radioactive iodine by the thyroid was then determined, as previously described, by serial counts taken over the thyroid isthmus with a shielded Geiger-Muller counter. A brass cap was placed over the window and in contact with the skin so that only gamma radiations were recorded.

By plotting the number of counts per second against the square root of the time in minutes, a straight-line relationship was found to exist with normally functioning thyroid glands. This relation was maintained quite well over a period of eight to twelve hours, except in those subjects which exhibited unusually rapid rates of uptake; in these instances there was a tendency for the points to fall below such a line towards the end of the day. The slope of this line has been termed the "accumulation gradient." Any antithyroid effect could be detected by the deviation of the gradient so that the plotted points were below where they would be if the straight-line relationship continued to hold.

After the ingestion of the I^{131} , counts were taken about every fifteen minutes until the course of the uptake seemed to be fairly well established. This usually required sixty to ninety minutes. The subject was then given the food to be assayed and instructed to eat to satiety in as short a time as possible. The total period of ingestion varied from one minute to two hours, the average being approximately forty minutes. Following the completion of the test meal, the subjects were free to go about their duties, returning for counts at one-hour intervals during the day. The final count was usually made between 4:30 and 5:00 P.M. In order to ascertain the effect of the test meal over as long a period as possible, the determinations on nine subjects were continued until 8:30 to 9:00 P.M. Following the final reading on the day of the test, the subjects were permitted to eat any food they desired but were asked to keep a list of all the material ingested (including liquids). A final reading was taken the next morning between 10:00 and 10:30 A.M. Except for a little coffee, tea, or water, no food was taken other than the test meal on the day of the experiment until after the final count in the afternoon.

The degree of inhibition of iodine uptake was arbitrarily graded in the following manner:

- 0—No inhibition. The course of the iodine accumulation did not deviate significantly from prediction.
- 1—Slight or questionable depression of the rate of uptake.
- 2—Moderate deviation from prediction but without complete inhibition.
- 3—Complete inhibition of the iodine uptake but for less than four hours.
- 4—Complete inhibition of uptake lasting more than four but less than twenty-four hours.
- 5—Complete inhibition of the iodine uptake lasting twenty-four hours.

The amount of radioactive iodine in the gland at the twenty-five-hour counting was compared with a graph which correlated the twenty-five-hour reading with the accumulation gradient of the preceding day in untreated normal subjects (Figure 1). The correlation seemed to hold fairly well within

relatively narrow limits in normal individuals. In the food experiments, a marked inhibition on the day of the test was often followed by a twenty-five-hour reading well below the average according to this graph. A slight or moderate depression of the gradient would often have no effect on the radioactivity present in the gland the next day.

It soon became apparent, however, that the twenty-five-hour count would sometimes be depressed although there had been no apparent antithyroid effect during the first day. Those who had a depression of their

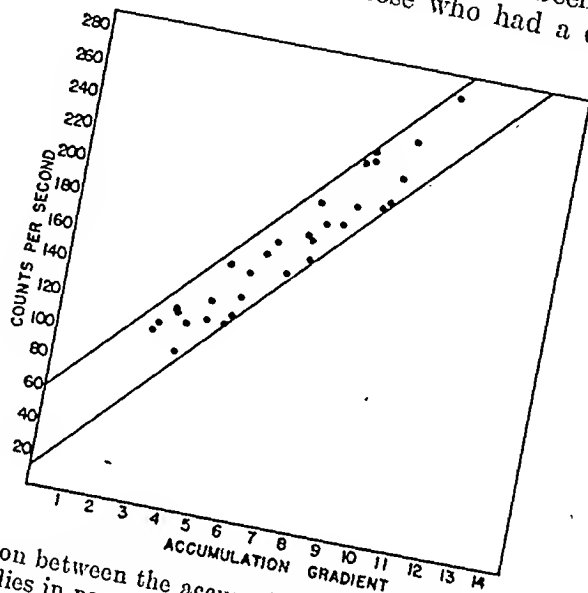


Fig. 1. The relation between the accumulation gradient on the first day of iodine uptake studies in normal, untreated subjects and the 25-hour count.

twenty-five-hour count without any apparent effect the day before had sometimes consumed considerable quantities of food suspected of having antithyroid properties. Consequently, the twenty-five-hour reading was of little value in assessing the degree of effect in these tests and was used only to differentiate between a grade 4 and 5 effect.

The test meal was limited to a single foodstuff in all except one of the experiments. No condiment was given during the first few tests; but, after it was established that it had no effect, table salt was used. A little butter was allowed with some of the cooked vegetables.

RESULTS

The following table lists the various foods tested and the grading of their effect by the arbitrary classification given above. There are three broad categories: Animal, Vegetable, and Mineral.

The products of plant origin are listed under their family names, arranged in alphabetic order. The members of the various families are listed in the first column alphabetically under their common names, with the scientific names in the second column. As the animal group consists of only a few members, these are listed only in the alphabetic order of their common names.

A total of 61 foods were tested in 100 different subjects. Most of those which showed more than a slight effect were tested at least twice, and one (rutabaga) was tried seven times. The subjects ingested the material in the raw state, when possible, since it was felt that activity might be destroyed by heat. If the raw foodstuff showed activity, it

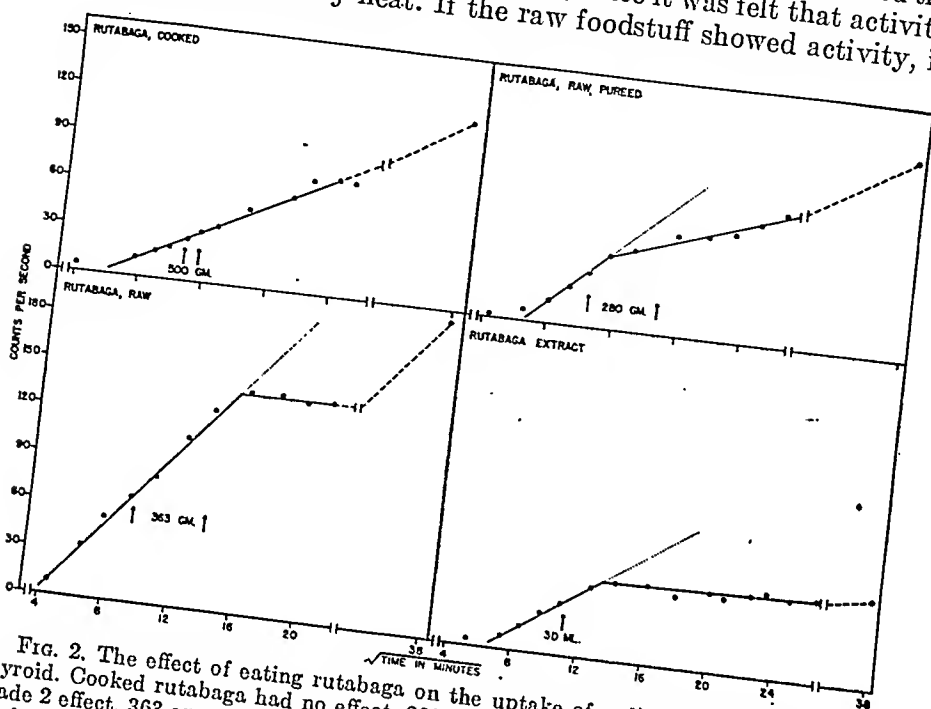


FIG. 2. The effect of eating rutabaga on the uptake of radioactive iodine by the thyroid. Cooked rutabaga had no effect. 280 gm. of the raw, pureed vegetable had a grade 2 effect. 363 gm. of raw chunks had a grade 3 effect. 30 cc. of a purified extract equivalent to 2617 gm. inhibited the uptake completely for 24 hours.

was usually tried after cooking, also. Some of the foods are so unpalatable in an uncooked condition, however, that no one would volunteer to eat them. Similarly, no one seemed anxious to try certain materials, such as lettuce, after cooking.

Since most of the positive goitrogenic effects shown by previous animal experiments seemed to be given by the Cruciferae, more tests were made among members of this group than of any other. Contrary to expectation, a high degree of activity was not found in cabbage. However, another member of the mustard family, rutabaga, was uniformly inhibitory. This vegetable has consistently given the strongest antithyroid effect. Turnips, closely related to rutabaga, possessed considerable activity also, but not to as marked a degree. The other members of the Cruciferae which were tested have so far proved inactive.

It is of interest that a definite inhibitory effect could be obtained from cabbage in only one instance. Since the goitrogenicity of this food has been quite definitely established for rats and rabbits, the

lack of a consistent effect on human beings may mean either that a considerable species difference exists in the response to this material or that the cabbage we obtained was relatively impotent.

Of the other families tested, the *Chenopodiaceae*, *Compositae*, *Cupuliferae*, *Juglandaceae*, *Leguminosae*, *Rosaceae*, *Rutaceae*, *Umbelliferae*, and *Vitaceae* showed definite evidence of activity among certain of their members. All the tested members of this family produced inhibition of the uptake of iodine by the thyroid. Beets and spinach

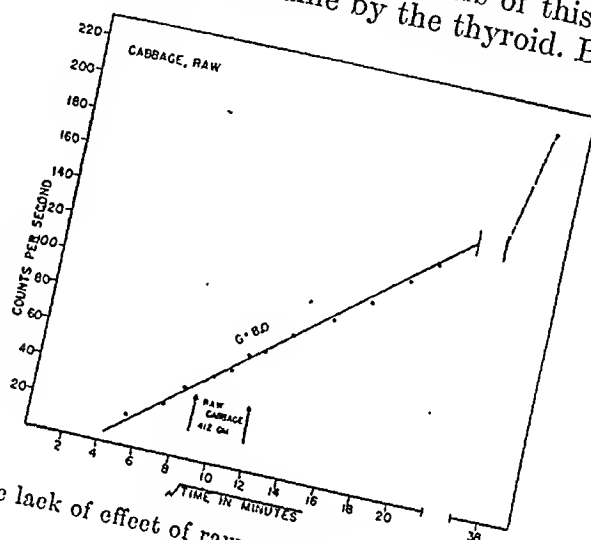


Fig. 3. The lack of effect of raw cabbage on the iodine uptake.

were both employed in the cooked state. In the one negative test given by spinach, only 322 gm. were eaten. Since this was less than half the amount consumed in the two positive tests of this foodstuff, the lack of effect in this instance might well be due to an insufficient concentration of the active principle in the body fluids.

Compositae. Raw lettuce, the only one of this group tested, had a slight effect in two instances. *Cruciferae*. As previously mentioned, the most active food we found, rutabaga, is a member of this family. This vegetable possessed marked antithyroid activity when ingested in the raw state in amounts varying from 280 to 363 grams. We were unable to obtain any effect when the rutabaga was cooked, however, other than a

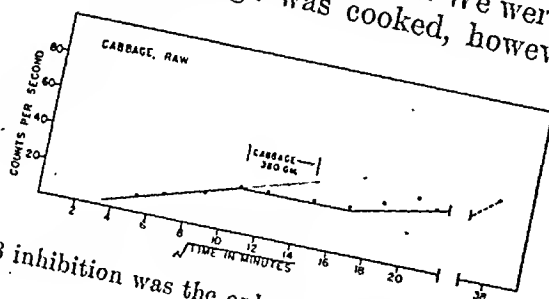


Fig. 4. This grade 3 inhibition was the only definite effect obtained from cabbage.

slight inhibition when 515 grams were consumed. The raw rutabaga was effective when eaten whole, as a puree, or when only the pressed, filtered juice was used. This indicated that the active principle was probably water-soluble. As a further confirmatory procedure, 30 cc. of a purified rutabaga extract, equivalent to 2617 gm. of the raw food, were taken by one subject. This resulted in a complete inhibition of the iodine uptake which lasted for twenty-four hours. Cooked broccoli and cauliflower showed no effect, nor did dry mustard in the small quantity of 0.9 gm. Radish also proved im-

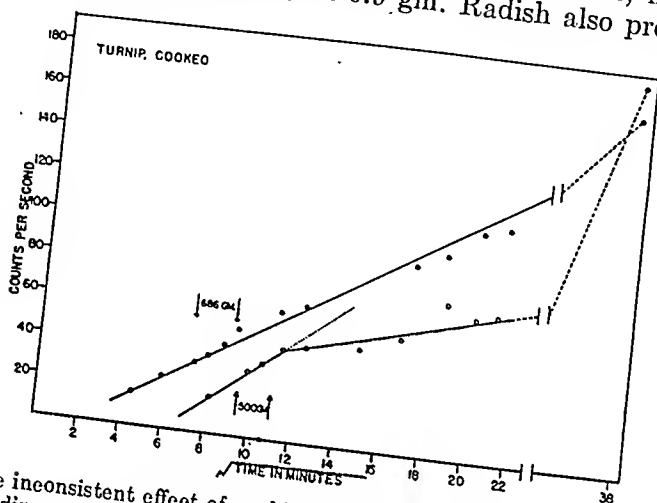


Fig. 5. The inconsistent effect of cooking. 686 gm. of cooked turnip did not inhibit the iodine uptake whereas a smaller meal of 500 gm. had a grade 2 effect.

tent. Raw turnip was definitely inhibitory in a dose of 441 gm. but had no effect when half that amount was taken. Cooked turnip depressed the uptake in one test when 500 gm. were eaten but had no effect in two other tests with 514 and 686 gm.

The largely negative findings in the case of cabbage have already been discussed.

Cupuliferae. The finding that only 186 gm. of raw filberts exerted a demonstrable inhibition suggests that this nut may possess a considerable degree of antithyroid activity. Unfortunately, we were unable to find a volunteer who could consume a larger quantity.

Juglandaceae. Walnuts showed evidence of activity comparable to that of the filberts.

Leguminosae. Several members of this group were found active: baked beans, string beans, peas, and peanuts. Cooking seemed to destroy the activity of the string beans, while it had no effect on that of the peas or peanuts. It is interesting that the baked beans were the only canned product in which we were able to demonstrate any activity. Black beans and lima beans were the only legumes tested which had no effect.

Rosaceae. The rose family also possessed active members. Raw pears and filtered pear juice showed moderate antithyroid activity, but no effect was obtained from canned pears. The latter, however, were ingested in a much smaller quantity. An alcoholic extract of pears, equivalent to 4400 gm. showed only a questionable degree of inhibition. Other evidence also indicated that alcoholic extraction was not an efficient method of obtaining the active principle.

The first test made on strawberries was quite striking. There was a complete inhibition of the iodine uptake which was maintained

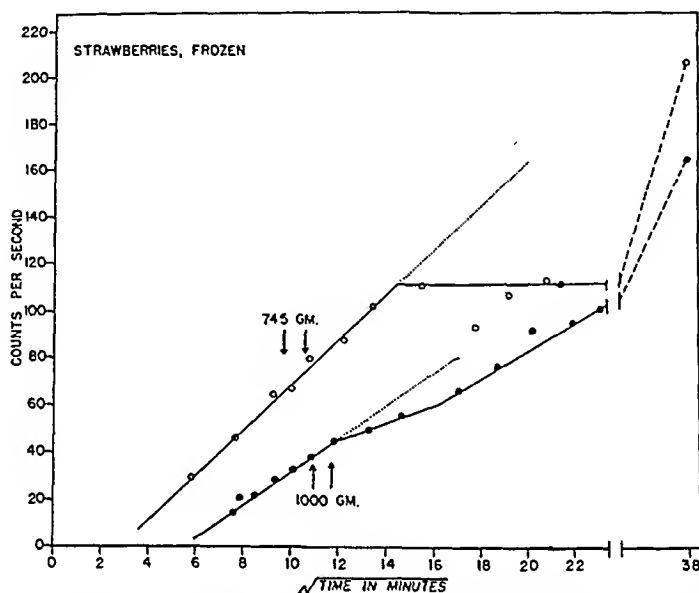


FIG. 6. 745 gm. of strawberries exerted a grade 4 inhibition in one subject while 1000 gm. of another batch had only a grade 2 effect.

until the final reading on the first day. However, the three subsequent tests of this product were not in agreement with the first. Two of these showed only a slight effect, while the third was negative. In each instance approximately the same quantity of strawberries was ingested. These differences in results may have been due either to a difference in the potency of the lots of fruit, or to a remarkable susceptibility of the completely inhibited subject. However, the previous test meal this subject had eaten (onions) had had a complete lack of effect on the accumulation gradient.

Raspberries, loganberries, and blackberries were inactive in the quantities tested, as were almond and apple. Peaches showed a definite effect, while apricots were only slightly inhibitory.

Rutaceae. Orange juice and grapefruit were moderately inhibitory but were taken in very large amounts. Tangerines had no activity.

Umbelliferae. Carrots proved quite active when eaten in adequate

quantity. A negative effect was obtained in only one of six instances from this foodstuff, and the 207 grams consumed in this test was approximately half that eaten in the other carrot experiments. Celery was responsible for a short period of complete inhibition when 630 gm. were eaten while 396 gm. had no effect.

Vitaceae. Fresh grapes had no activity, but when eaten in the form of raisins they caused deviation of the gradient although less than one-third of the amount taken fresh was consumed. This difference in potency was most likely due to the dissimilarities of the varieties used. The fresh grapes were Tokay, while the raisins were Thompson seedless.

Animal. Of the products tested which can be classified as animal, milk had the most consistent antithyroid effect. The inhibition produced was never marked but seemed definite, both as pasteurized

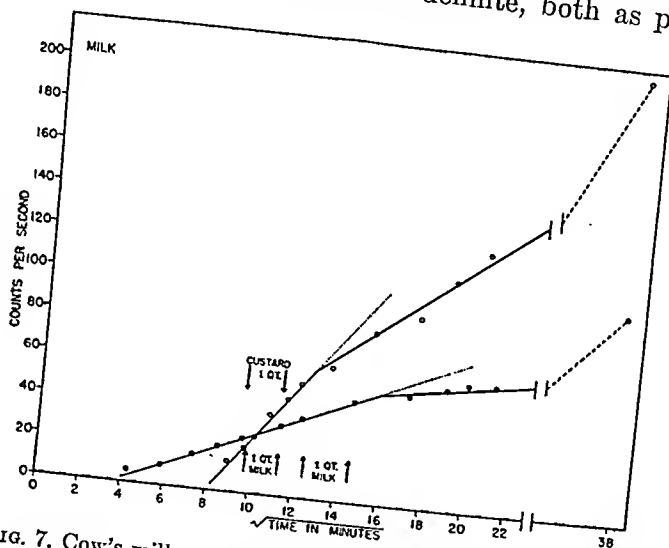


Fig. 7. Cow's milk, either as baked custard or as uncooked, pasteurized milk, gave a grade 2 inhibition.

milk and as baked custard. Of the five tests carried out on milk or milk products, only two negative results were obtained. One of these was with processed American cheese where only a small quantity (160 gm.) was consumed. The other negative test in this group followed the consumption of three quarts of pasteurized milk. Since this would seem to be an adequate amount, one can only state that milk, as such, had a positive effect in two out of three instances. The active principle which may be contained in this food is apparently heat-stable as neither baking nor pasteurization seemed to destroy the antithyroid effect.

Raw oysters and raw clams also depressed the iodine uptake. No other seafood tested showed any such inhibition. While it is possible

that the depression of the accumulation gradient by oysters and clams may have been due to the high-iodine content they are known to possess, the absence of activity from the other iodine-rich seafood is difficult to correlate with such an hypothesis. It is possible, of course, that boiling a marine animal will leach out enough iodine to reduce the concentration of this element below an effective level. In line with this thought, it might be noted that the oysters and clams were the only ocean residents eaten raw.

Because several observers (Remington, 1937, Hou, 1940) have indicated that liver may be a potent goitrogen in the rat, this substance was tested in man also. As yet only one such test has been made. One pound of broiled beef liver gave evidence of a definite, though slight, thyroid inhibition.

Mineral. In some of the tests with a grade 1 inhibition it was rather difficult to state categorically whether or not the effect was significant. Because it was possible that some of these questionable effects might be due to inert materials, a test was made to determine what result would follow the administration of a large amount of salt. 28.4 gm. of sodium chloride were given orally with no demonstrable depression of the iodine uptake. This result indicated that any possible displacement of iodide by the chloride ion had no significance in our tests as no meal given had a salt content which approached this amount. Since three liters of water would have to be drunk by this subject to maintain isotonicity of the body fluids, this test also indicated the lack of inhibition by a large, inert fluid intake.

Mixed Food. Since it seemed unlikely that meals would very frequently be limited to a single food, a test was made which combined carrots, rutabaga, lettuce, pears, and milk. This resulted in a 1-2 degree inhibition, indicating that such combinations could also have antithyroid activity.

DISCUSSION

These results suggest that there are materials in certain foods which are capable of inhibiting the uptake of iodine by the human thyroid. Of the products thus far tested, rutabaga consistently possessed greater activity than any other. On the whole, those foods belonging to the vegetable kingdom seem more active than animal products.

Some of the tests which yielded only grade 1 effects were difficult to interpret and probably were of no significance; however, those that were stronger than this probably represent a true inhibition of the thyroid. Distention of the stomach by a bulky mass or dilution of the body fluids by the addition of large amounts of liquid probably did not significantly alter the rate of thyroid iodine accumulation. For instance, the three quarts of milk and the 1263 gm. of banana that were ingested had no effect although they were as large in quantity as any other food tested. The negative effect from sodium

chloride previously cited would lend support to such an inference.

Although rutabaga had the strongest antithyroid effect, activity was by no means limited to the Cruciferae. Among the Rosaceae, for instance, pears, strawberries, and peaches seemed to be quite active. Whether the material responsible for this effect was the same in each instance or whether, as seems more likely, different compounds were responsible in different foods can only be answered by the isolation and identification of the active principle in each case.

It is curious that the activity of some foods, e.g. rutabaga and pears, seemed to be destroyed by cooking while the activity of others, such as peas and peanuts, was unaffected by heat. Since the antithyroid ingredient seemed to be water soluble, perhaps the heating of some of these foods in water extracted a good proportion of the activity. The cooking juices were usually not eaten in these tests so the material ingested may have thus lost a good deal of its inhibiting power. Peanuts, on the other hand, were roasted and there was therefore no opportunity for the activity to be extracted.

Of course, some of the vegetables which retained their activity when cooked were also heated in water. In these cases there might conceivably be a difference in solubility of the active material or some mechanical interference with the water's ability to reach the food pulp. Peas and beets, for example, were cooked whole and the integrity of the outer coat might have at least partially obstructed the access of water to the interior. In other instances where such an explanation could not hold, the juices may have been eaten as well as the pulp. It is also possible that if the goitrogens in different foods are not the same, heat may destroy some and not others.

A pertinent question is whether the iodine content of the active foods contributed to the depressed rate of accumulation of radioactive iodine. Tests with various doses of potassium iodide have shown that approximately 1 mg. would be required to induce a perceptible effect. Although the iodine content of the foods used in these experiments was not determined, the extensive literature on the subject indicates that most of the test meals were very low in iodine. The sea foods were an exception and the values quoted by McClendon (1939) indicate that several milligrams of iodine may have been present. The fact that positive effects were observed only with raw oysters and clams might indicate that the iodine present in the other sea foods was not in the form of iodide or not in a form which was readily available for absorption by the thyroid. Another possibility, that boiling might leach out the iodide from the sea food, has already been mentioned.

The findings reported here appear to have some significance in relation to the development of simple goiter. If the diet consistently contained foods high in antithyroid activity, it is possible that goiter would eventually result. A planned experiment to test this possibility has not been tried, but cases have been observed of goiter

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		State	Quantity	Effect	
ANIMAL					
Beefsteak	Venus mercenaria	raw	448	0	
Bonita		canned	308	0	
Cheese		American, pasteurized	160	0	
Clams		raw	2 dozen	1	
Custard	Homarus sp.	baked	1000	1	
Ice cream		frozen	1 quart	0	
Liver		boiled	1 pound	1	
Lobster	Ostrea virginica	pasteurized	600	0	
Milk		pasteurized	2 quarts	1	
Milk		pasteurized	2 quarts	2	
Milk	Crangon sp.	raw	3 quarts	0	
Oysters		raw	1 pint	2	
Oysters		canned	1 pint	0	
Sardines		boiled	218	0	
Shrimp			400	0	
AGARICACEAE					
Mushrooms	Agaricus campestris	cooked	570	0	
ARECACEAE					
Date	Phoenix dactylifera	dried	400	0	
BROMELLIACEAE					
Pincapple	Ananas comosus	canned	1200	0	
CHENOPODIACEAE					
Bcets	Beta vulgaris Spinacia oleracca	cooked	502	1	
Spinach		cooked	322	0	
Spinach		cooked	808	2	
Spinach		cooked	900	1	
COMPOSITAE					
Lettuce	Lactuca sativa	raw	284	1-2	
Lettuce		raw	299	1	
CRUCIFERAE					
Broccoli	Brassica oleracea botrytis	cooked	263	0	
Cabbage	Brassica oleracea	raw	260	0	
Cabbage		raw	412	0	
Cabbage		raw	380	1	
Cabbage		cooked	380	3	
Cauliflower			560	0	
Mustard	Brassica oleracea botrytis	dry	358	0	
Radish	cauliflora	raw	363	0	
Rutabaga	Brassica alba	raw, pureed	280	3-4	
Rutabaga	Raphanus sativus	juice, raw	313	2	
Rutabaga	Brassica napobrassica	purified	30 ee.	2	
Rutabaga		extract	(equiv. 2617 gm.)	5	
	Brassica rapa	cooked	500	0	
		cooked	515	1	
		cooked	290	0	
		raw	234	0	
		raw	441	0	
		cooked	514	2	
		cooked	686	0	
		cooked	500	2	

ANTITHYROID EFFECT OF FOODS

		State	Quantity	Effect
<i>CUCURBITACEAE</i>				
Cucumber	Cucumis sativus	raw		0
Honeydew	Cucurbita maxima	green, frozen	590	1
Squash, banana		cooked	456	0
			400	0
<i>CUPULIFERAE</i>				
Filbert	Corylus avellana	raw	186	1
<i>GRAMINEAE</i>				
Corn	Zea Mays	frozen	475	0
Rice	Oryza sativa	boiled	350	0
Rye	Secale cereale	bread	250	0
<i>JUGLANDACEAE</i>				
Walnut	Juglans regia	raw	330	1-2
<i>LEGUMINOSAE</i>				
Beans, black	Dolichos lablab	cooked	334	0
Beans, baked	Phaseolus limensis	canned	479	1
Beans, lima	Phaseolus vulgaris	cooked	290	0
Beans, string		raw	389	0
Beans, string	Pisum sativum	cooked	633	1-2
Peas		raw	880	0
Peas		cooked	443	1-2
Peanut	Arachis hypogaea	raw	200	1
Peanut		roasted	218	1
<i>LILIACEAE</i>				
Onion	Allium cepa	raw	93	0
<i>OLEACEAE</i>				
Olive	Olea europaea	pickled, green	200	0
<i>ROSACEAE</i>				
Almond	Prunus Amygdalus	raw	132	0
Apple	Malus pumila	raw	689	0
Apricot	Prunus Armeniaca	dried	320	1
Blackberry	Rubus spp.	frozen, raw	825	0
Loganberry	Rubus loganobaccus	frozen, raw	761	0
Peach	Prunus Persica	frozen, raw	810	0
Pear	Pyrus communis	raw	1455	2
Pear		juice, raw	803	2
Pear		raw	1044	2
Pear		canned	530	1
Pear		alcoholic	equiv. 4.4 kg.	0
Peanut		extract		1
	Rubus idacus	frozen, raw	447	0
	Fragaria spp.	frozen, raw	746	5
		frozen, raw	860	0
		frozen, raw	1000	1
		fresh	791	1
<i>RUTACEAE</i>				
Grapefruit	Citrus grandis	raw	1326	2
Orange	Citrus aurantium	juice, raw	1179	1
Tangerines	Citrus reticulata	raw	430	0

GREER AND ASTWOOD				Volume 4
		State	Quantity	Effect
<i>SCITAMINAE</i>				
Banana	Musa sapientum	raw	1263	0
<i>SOLANACEAE</i>				
Green pepper	Capsicum annum var.	raw	244	1
Potato	Solanum tuberosum	raw	381	0
Potato		boiled	692	0
Tomato	Lycopersium esculentum	raw	600	0
Tomato		juice, fresh	855	0
<i>UMBELLIFERAE</i>				
Carrot	Daucus carota	raw	416	1
Carrot		raw	595	1-2
Carrot		raw	378	2-3
Carrot		raw	352	3
Carrot		cooked	207	0
Celery	Apium aveolengrs	raw	554	1
Celery		raw	630	3
		raw	396	0
<i>VITACEAE</i>				
Grapes	Vitis vinifera	fresh	680	0
Grapes		dried	275	1-2
<i>MIXED MEAL</i>				
Carrots, rutabaga, lettuce		raw	420	1-2
Pears		raw	311	
Milk		pasteurized	3 glasses	
<i>MINERAL</i>				
Sodium ehloride		tablet	28.4	0

developing in patients who were vegetari-
cravings for certain foods
notable that

developing in patients who were vegetarians or who suddenly had cravings for certain foods, such as lettuce. Certainly it seems indisputable that goiter can be caused in animals by a high dietary intake of certain foodstuffs. Not only is this true in the laboratory, but also among livestock, as in the epidemics described by Hercus and Purves of goiter in sheep which were fed on turnip during the winter.

Of course, the amount of food consumed in the above experiments was unphysiologically large in most instances. However, some foods, such as peanuts, rutabagas, and filberts, exerted an inhibition when relatively small quantities were eaten.

Since there are many people who habitually consume considerable amounts of foods that have been found to possess activity and who do not develop goiters, a marked variation in the response to goitrogenic stimuli probably exists. Clinical experience has shown this to be the case in the requirements for antithyroid medication in thyrotoxicosis.

Probably there is a good deal of variation in the potency of foods at different times also. Webster (1932) reported that the goitrogenic activity of cabbage obtained from the same source became much more marked with the approach of the winter months.

The antithyroid material consumed by the vast majority of people eating such foods would probably not be sufficient to have any visible effect on the thyroid. Only if the major portion of one's dietary intake were limited to goitrogens would thyroid hyperplasia be likely to result. However, under conditions of iodine deficiency, the added stimulus of goitrogenic food might produce hyperplasia in a thyroid gland which was just able to produce sufficient hormone to satisfy the metabolic requirements of the body, while still retaining its normal size.

The nature of the active antithyroid compounds in these food-stuffs is unknown. Active material could be extracted with ether from the clear aqueous extract of rutabaga. When the resulting ether extract was dried, the active material would dissolve in a small volume of water. Such a purified extract, equivalent to 2617 gm. rutabaga, completely inhibited the uptake of iodine for a full twenty-four hours when ingested.

SUMMARY

The antithyroid effect of sixty-one different foods was tested in one hundred different subjects by using radioactive iodine. Of these, rutabaga was found to possess the greatest antithyroid activity. Vegetables were more active than animal products and the most potent vegetable families were the Chenopodiaceae, Compositae, Cruciferae, Cupuliferae, Juglandaceae, Leguminosae, Rosaceae, and Umbelliferae. Cow's milk, beef liver, and oysters also showed some activity.

The active principle of rutabagas could be extracted from a watery solution with ether and was contained in the water-soluble portion of this dried ether extract.

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NOTES AND COMMENTS

HYPERVITAMINOSIS A AND THE DISTRIBUTION OF BODY IODINE

THE RESULTS of numerous investigations on vitamin A and thyroid physiology (Drill, '43; Simkins, '47) have uncovered relationships which require further investigation. Experiments dealing with the effects of thyroxine administration, or of thyroidectomy on vitamin A storage will not be considered in this paper; these studies have been adequately reviewed in the publications cited above. The effects of vitamin A-deficiency and, especially, of hypervitaminosis A on thyroid function are of primary interest to the present discussion. Briefly, avitaminosis A produces hypertrophy of the thyroid in female and atrophy of the thyroid in male rats (Coplan and Sampson, '35), and an increase in thyroid weight (Coplan and Sampson, '35; Lipsett and Winzler, '47); some of the thyroid follicles exhibit the changes of colloid goiter (Lipsett and Winzler, '47). Conversely, large doses of vitamin A reduce thyroid weight (Sadhu and Brody, '47) and reduce the colloidal material in the thyroid follicles (Sherwood, et al, '34).

The histological changes mentioned are accompanied by marked effects on iodine storage and utilization. The inability of the thyroid to regulate iodine normally, in the present of A-deficiency, has been amply demonstrated (McCarrison, '30; Mitzkewitsch, '34; Coplan and Sampson, '35; Lipsett and Winzler, '47). A relationship between iodine metabolism and vitamin A has also been indicated by studies utilizing large oral doses of the vitamin (Lipsett and Winzler, '47; Simkins, '47; Truscott and Sadhu, '48a).

It is conceivable that the effects of vitamin A on the thyroid and on iodine metabolism are inseparable factors and are mediated by a pituitary-thyroid relationship. This is suggested by the work of several investigators (Elmer, et al, '35; Fellinger and Hochstadt, '36; Schulze and Hundhausen, '39; Truscott and Sadhu, '48a). Such studies have indicated, directly or indirectly, that hypervitaminosis A exerts an inhibitory action on the secretion of the thyrotrophic hormone of the pituitary. The following experiments were undertaken to gain some insight into the mechanism by which vitamin A might exert this action.

MATERIALS AND METHODS

The animals used in this investigation were adult male albino rats, which were divided into one control and two experimental groups. The animals in the first experimental series received a daily oral dose, per rat, of 15,000 I.U. of vitamin A administered as Afaxin.¹ The second experimental group received a daily oral dose of 20,000 I.U. per rat, given as percomorph oil. The length of the dosage periods were 14 days for the first and 18 days for the second group.

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¹ Vitamin A concentrate furnished by the Winthrop Chemical Co.

The rats were sacrificed at the end of the experiment, and the protein-bound iodine of the tissues was determined by the method of Salter and McKay ('44).² Vitamin A values were obtained by the antimony trichloride method, with the use of a Lumetron photoelectric colorimeter.

RESULTS

Hepatic iodine was markedly decreased in the hypervitaminotic animals (table 1), especially in those receiving the smaller dose of the vitamin; the magnitude of the iodine decrease, however, was not proportional to the amount of vitamin A administered.

The low level of hepatic iodine made it advisable to determine whether this formed part of a general depression of iodine content in other tissues of the body. Because the iodine content of the pituitary, and particularly of skeletal muscle is normally high, it was believed that any general alteration in iodine distribution would be reflected in the iodine content of these tissues. In contrast to the decrease in hepatic iodine, both pituitary and muscle iodine was significantly increased. It appeared unlikely, therefore, that the iodine level in the liver was due to a general lowering in the iodine content of body tissues.

Protein-bound serum iodine was increased and was directly related to the dosage level of vitamin administered. There was an increase of 17.1% and 34%, respectively, in the Afaxin- and percomorph oil-fed animals; a statistically significant change however, was present only in the percomorph rats. In agreement with the results obtained in liver analyses, the magnitude of the changes in serum iodine was not proportional to the dosage level of the vitamin.

TABLE 1

	322	185	292
Body weight (gm.)	20	12	18
Number of animals	6.18	5.03	4.03
Thyroid weight (mg. %)	3.21 \pm 0.12	3.76 \pm 0.92	4.33 \pm 0.76*
Serum iodine (γ /100 ml.)	90.4 \pm 3.3	82.1 \pm 4.5†	67.3 \pm 4.8*
Thyroid iodine (γ /100 mg.)	0.201 \pm 0.021	0.097 \pm 0.013*	0.120 \pm 0.05*
Hepatic iodine (γ /gm.)	0.2243 \pm 0.586	—	0.3443 \pm 0.213*
Muscle iodine (γ /gm.)	0.130 \pm 0.009	0.253 \pm 0.015*	0.226 \pm 0.017*
Pituitary iodine (γ /100 mg.)	85.0	260	420
Serum vitamin A (I.U. %)	408.0	7188.5	11009.5
Hepatic vitamin A (I.U. %)			

* $p < .01$.† $p < .05$.

These results suggested that there was a decreased destruction of thyroxine by the liver, thus leading to an increase in circulating thyroxine. It would be expected, therefore, that thyrotrophic hormone secretion would be depressed with an eventual decrease in both weight and iodine content of the thyroid gland. Consequently it was of interest to observe that, in addition to the increase in pituitary iodine, there was a reduction in thyroid weight and iodine content; furthermore, these changes were related to the amount of vitamin administered, the lower values existing in the group receiving the larger amount of the vitamin.

² The authors wish to thank Dr. Wm. T. Salter for placing laboratory facilities at their disposal for the iodine determinations.

Ineffective storage and distribution of the administered vitamin in the experimental groups remained a possibility. The liver and blood levels of vitamin A were determined in an attempt to ascertain whether the above possibility could be ruled out. It will be noted (table 1) that the high values obtained fall within the expected range and are directly related to the amount of vitamin fed.

DISCUSSION

The reduction in thyroid iodine and thyroid weight, following oral administration of large amounts of vitamin A, supports the concept that the vitamin may exert an inhibitory action on thyrotrophic hormone secretion (Elmer, et al, '35; Fellingner and Hochstadt, '36; Schulze and Hundhausen, '39; Truscott and Sadhu, '48a). Certain investigators (Belasco and Murlin, '40; Sadhu and Brody, '47) have suggested that the iodine in the thyroxine molecule is picked up by the double bond of the vitamin; the vitamin A-iodide thus formed may depress the secretion of thyrotrophic hormone and result in a decrease in thyroid size. Sadhu ('48), however, observed that this action was more marked when vitamin A was fed alone than when it was administered in conjunction with iodine in the diet. He concluded, therefore, that the vitamin does not reduce thyrotrophic hormone secretion by the formation of a vitamin A-iodide or -thyroxine like compounds.

A possible explanation of the above results is suggested by the data presented in this paper. Following oral administration of large doses of vitamin A, there appears to be a lessened destruction of thyroxine by the liver; it has been demonstrated (Truscott and Sadhu, '48b) that this is not due to a displacement of thyroxine iodine from hepatic tissue by the accumulation of excessive amounts of the vitamin in the reticuloendothelial cells. The consequent hyperthyroxinemia affects the pituitary depressing the secretion of thyrotrophic hormone; this is indicated by the high values of serum and pituitary protein-bound iodine obtained in these animals. With depression of thyrotrophic hormone secretion, there is the observed decrease in protein-bound iodine and in weight of the thyroid gland.

SUMMARY

The distribution of body iodine was studied in albino rats receiving large oral doses of vitamin A.

Hypervitaminosis A was accompanied by a decrease in protein-bound iodine in liver and thyroid, and by an increase in protein-bound iodine in serum, pituitary and skeletal muscle.

It is suggested that these results are due to a decreased hepatic destruction of thyroxine, with a consequent hyperthyroxinemia; the latter depresses thyrotrophic hormone secretion, producing the observed decrease in both thyroid weight and in protein-bound iodine in the thyroid.

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RENAL FUNCTION IN THE ADRENALECTOMIZED RAT¹

DESPITE the voluminous literature concerning the effects of adrenalectomy in a wide variety of species, surprisingly few attempts have been made to assess the immediate effects on renal function. Talbott et al (1942) studied renal function in patients with Addison's disease and in patients with adrenal insufficiency secondary to pituitary pan hypofunction. They reported that while the ordinary clinical tests showed no aberration, more precise quantitative procedures, using inulin and diodrast, showed impairment of all measured functions. This impairment included the glomerular filtration rate, renal plasma flow and tubular excretory as well as reabsorptive mass. More recently, Sanderson (1948) has studied renal function in four cases of untreated Addison's disease not in crisis, and noted a similar depression of all functions.

White, Heinbecker and Rolf (1947) studied the inulin and diodrast clearance in two adrenalectomized dogs. They observed that mild adrenal insufficiency, in which the blood volume, NPN, and sodium and potassium levels were all normal, was accompanied by a great decrease in C_D and T_{MD} with a lesser fall in C_{IN} . These changes were more completely restored by the implantation of desoxycorticosterone acetate pellets than by adrenal cortical extract. The effect of saline maintenance alone was not studied.

In view of the azotemia which may be observed in patients with Addison's disease, it seems important to ascertain more definitely just how far the kidney may play a contributory rôle. Experiments on rats were undertaken to determine the immediate effects of adrenalectomy with saline maintenance on renal function in this animal.

EXPERIMENTAL

Three separate experiments were carried out to assess renal function 1, 4, and 6 days after bilateral adrenalectomy. For each experiment, mature hooded male rats, weighing approximately 300 grams were selected. For the first two experiments, the animals were slowly adapted to tube feeding and were maintained at constant intake for at least one week prior to operation.

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The animals of the third experiment received Purina Fox Chow ad lib. Adrenalectomy was performed through bilateral lumbar incisions and following this, both control and adrenalectomized animals were maintained with 1% saline as drinking water while tube feeding was continued. For the first experiment, the control animals were subjected to a sham adrenalectomy. As this procedure was without apparent effect on renal function as determined one day later, it was not used in subsequent control groups. Renal function was determined using inulin and sodium p-aminohippurate by the method previously described for the comparative investigation of groups of rats (Friedman, Polley and Friedman, 1947). Control and test animals were studied at the same time.

TABLE 1

Time post adrenalectomy	Expt. 1		Expt. 2		Expt. 3	
	1 day		4 days		6 days	
	Sham operated	Adrenalectomized	Untreated control	Adrenalectomized	Untreated control	Adrenalectomized
C_{IN} cc./100 cm. ² /min.	0.24 ±0.03	0.30 ±0.03	0.23 ±0.03	0.23 ±0.05	0.27 ±0.04	0.22 ±0.04
C_{PAH} cc./100 cm. ² /min.	2.14 ±0.31	1.46 ±0.34	2.13 ±0.33	1.59 ±0.22	2.12 ±0.31	1.92 ±0.27
Tm_{PAH} mgm./100 cm. ² /min.	0.107 ±0.006	0.067 ±0.012	0.108 ±0.010	0.088 ±0.021	0.114 ±0.010	0.110 ±0.015
FF as %	11.3 ±2.5	21.8 ±6.1	11.2 ±1.6	15.4 ±6.0	12.6 ±1.4	11.7 ±2.2
C_{PAH}/Tm_{PAH}	19.8 ±2.1	22.2 ±6.8	19.7 ±2.7	18.2 ±1.6	18.7 ±2.7	17.4 ±1.0
No. of animals	4	4	7	7	7	7
Average body weight	307	318	330	318	290	284

Table 1 presents the findings. It is apparent that kidney function as determined in the three control groups, made up of different animals studied at entirely different periods, did not vary, and hence represents a good standard of comparison for the test animals. The spread of data is here rather less than usually observed with this method, probably due to the standardized feeding as well as to the large size of the animals. It is also noteworthy that the normal values shown here, especially that of C_{IN} , are lower than those observed in albino rats of the Wistar strain which we have previously reported. The data indicate that this is in part referable to a strain difference, and in part to the larger size of the animals used here.

One day after adrenalectomy a marked fall in renal plasma flow, C_{PAH} , was observed, together with a parallel decrease in the functioning tubular excretory mass, Tm_{PAH} . This was not accompanied by any decrease in the glomerular filtration rate, C_{IN} , which, in fact, was slightly elevated above the control value.

Four days after adrenalectomy renal function was still depressed, although to a lesser extent. Again, C_{PAH} and Tm_{PAH} were the functions most affected, while C_{IN} remained unchanged.

Six days after adrenalectomy renal function was essentially normal.

Repeat experiments were performed on groups of Wistar albino rats. The same initial depression followed adrenalectomy, and when the animals were

maintained with 1% saline as drinking water renal function was restored to normal at 6 days.

DISCUSSION

It is evident that immediately after adrenalectomy renal function is seriously impaired in the rat. The pattern of derangement is essentially that observed in the dog by White, Heinbecker and Rolf (1947) except that in their experiments C_{IN} was reduced, although to a lesser degree than the other functions studied. This need not represent an absolute difference between the species since the modifications in procedure necessary for estimating renal function in the rat impose limitations on the validity of such an inter-species comparison.

If the animal survives adrenalectomy and is maintained on saline only, renal function becomes essentially normal six days after operation. The fourth day after adrenalectomy appears to represent an intermediate period, since, as indicated by the spread of the data, some animals are approaching normality more rapidly than others.

The results obtained in the control animals indicate the necessity of mentioning the strain of rat employed in reporting clearance values in this species.

SUMMARY

One day after adrenalectomy in the rat (hooded and Wistar albino) there is a marked fall in C_{PAH} and Tm_{PAH} , while C_{IN} remains unaffected, as revealed by clearance studies using inulin and sodium p-aminohippurate.

Renal function in the adrenalectomized rat maintained with saline becomes essentially normal six days after operation, an intermediate phase in the restorative process occurring at four days.

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FAILURE OF MASSIVE DOSES OF ESTROGEN TO PROMOTE GROWTH OF ENDOMETRIAL COILED ARTERIOLES

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THE administration of massive doses of estrogens to monkeys has been demonstrated to produce squamous metaplasia of the endocervical mucosa and cystic hyperplasia of the endometrium. The present report is made to direct attention to the fact that estrogenic stimulation of this magnitude can induce excellent proliferative activity of endometrial glands and stroma without proliferation of endometrial coiled arterioles. In addition, further observations in reference to cervical epidermidization and endometrial hyperplasia have been made.

OBSERVATIONS

Six rhesus monkeys (*Macaca mulatta*) were given 25,000 I.U. of Amniotin² a day from February 11 through March 8 with the exception of No. 182 which was treated through March 4 only. They were all mature female castrates, resident in the colony of the Department of Embryology for several years. They had been employed during the previous six months in a series of estrogen and estrogen-progesterone withdrawal studies, in the course of which they demonstrated the expected patterns of menstruation-like bleeding.

Animal No. 182 was found moribund on March 5, and it was therefore killed. At autopsy no gross abnormalities were found. The reproductive organs were removed *en bloc* and fixed in formalin. Histologic sections were studied with the following findings.

Bladder and urethra: considerable cornification of the mucosa at the urethral orifice.

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² The Amniotin in oil (50,000 I.U. per cc.) was generously supplied by E. R. Squibb and Sons.

Vagina: deeply cornified.

Cervix: there is epidermidization of the apices of several endocervical glands and a patch of squamous metaplasia in the midportion of the cervical canal. These areas contrast sharply with the normal columnar "picket" epithelium, as can be seen in Fig. 1.

Uterus: there is a good response of the stroma and glands, corresponding to rhesus endometrium of about the 12th day of a 28-day cycle, without any evidence of hyperplastic activity. Only one coiled arteriole is found in a large number of serial sections of the entire fundus and this manifests little proliferative activity. The other two arterioles found are of the basal arteriole type. These features may be seen in Fig. 2.

The remaining five animals withstood the series of injections without disturbance of health. Each also developed a bright red sex skin without any remarkable excess in the amount of vaginal desquamation. The pattern of withdrawal bleeding is indicated in Table I.

TABLE 1. UTERINE BLEEDING IN FIVE CASTRATE RHESUS MONKEYS GIVEN 25,000 I.U. OF AMNIOTIN IN OIL DAILY FOR 26 DAYS (Feb. 11-Mar. 8)

Dates	Time from withdrawal to bleeding	Duration
No. 611 Mar. 15-20	7 days	6 days
No. 723 Mar. 16-22	8	7
No. 726 Mar. 15-19	7	5
No. 749 Mar. 17-19	9	3
No. 754 Mar. 14-20	6	7

Two intact rhesus monkeys were given 50,000 I.U. of Amniotin a day from May 12 to June 10, except on the four intervening Sundays, a total of 26 days.

Animal No. 144 had had cyclic bleeding throughout the preceding winter and spring. It did not manifest withdrawal bleeding in the twenty days following termination of the series of injections.

Animal No. 166, in the 1946-47 season, had bled starting November 21, December 18, January 11, January 31 and May 1. On May 9 it was noted to have an upper abdominal mass. On June 11 the animal was killed and autopsy revealed no gross abnormalities except for the mass, which was the liver. This organ, which weighed 950 gms., was the site of an atypical type of amyloid infiltration. The microscopic findings in the lower urinary and the reproductive organs were as follows:

Bladder, urethra and vagina: identical with the findings in No. 182.

Cervix: there is a most extensive epidermidization of the endocervical mucosa, in some cases involving entire glands, with sheets of squamous epithelium in the endocervical canal, undermining and replacing the "picket" epithelium. This may be seen in Fig. 3.

Uterus: there is a follicular response similar to that of No. 182. Study of numerous sections of the entire fundus fails to reveal any coiled arterioles.

DISCUSSION

Since the striking demonstration of the cyclic alterations of the endometrial coiled arterioles of the rhesus monkey by Daron (1936), it has been assumed by most students of menstruation that growth



FIG. 1. Endocervical canal from the mid-cervix of No. 182. Hematoxylin and eosin preparation $\times 150$. Normal "picket" epithelium is replaced at the right by a sheet of squamous cells. In the lower center the apices of two glands are replaced by squamous cells.

and differentiation of these vessels is always coordinated with the growth and differentiation of the endometrial glands and stroma. The studies of Markee (1940) and Kaiser (1947a) indicate that this is indeed the case in the normally menstruating intact animal. There has, however, been no detailed study of the appearance of endometrial coiled arterioles in animals with menstrual abnormalities or in castrated animals subjected to hormonal stimulation. Phelps (1947), making observations on intraocular endometrial transplants in castrated animals, has recently reported that the pattern of arteriolar growth response to hormone stimulation is significantly affected by the previous hormonal experience of the animal.

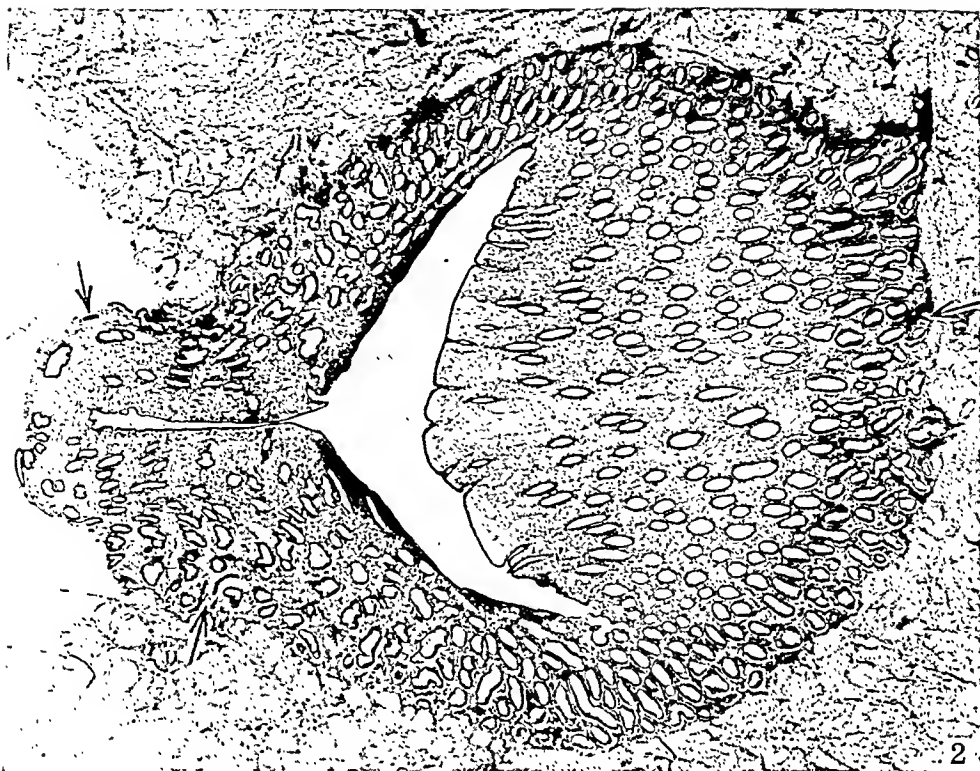


FIG. 2. The mucosa of the entire fundus uteri of No. 182 is enlarged 15 X. The only arterioles found are inked in for emphasis and indicated by arrows. One of these, at the lower left, is a poorly developed endometrial coiled arteriole. The other two are basal arterioles.

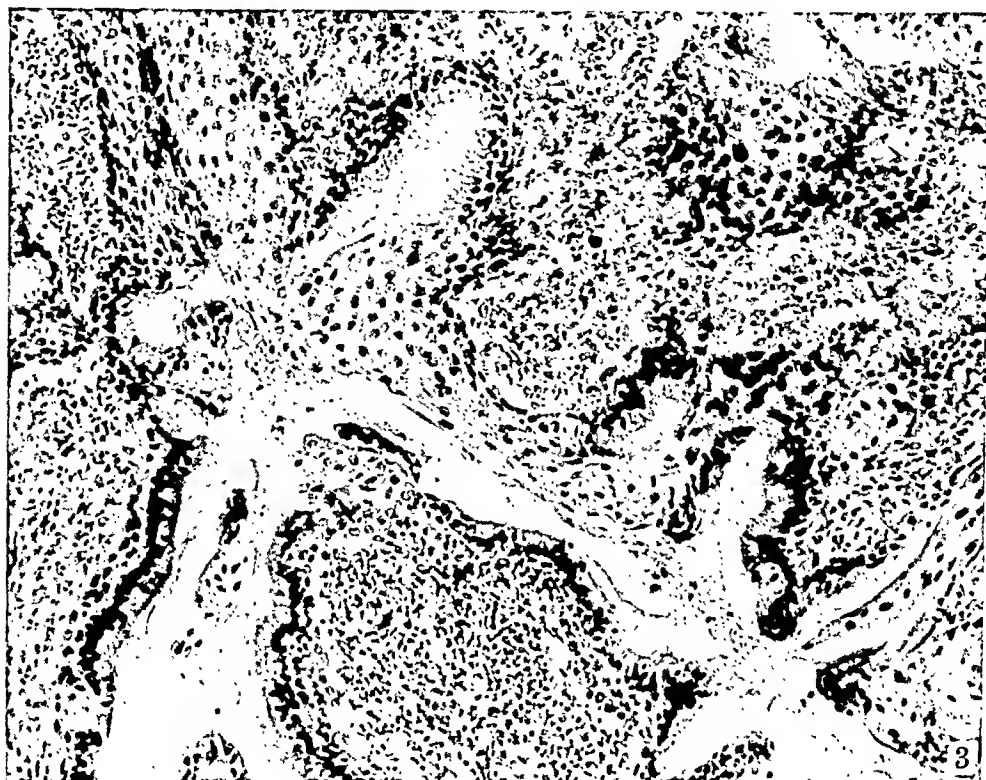


FIG. 3. Endocervical canal from the mid-cervix of No. 166. Hematoxylin and eosin preparation X150. The squamous metaplasia is most extensive.

In the present experiments with massive estrogen administration, an excellent proliferative response of endometrial glands and stroma has been observed, while the absence of coiled arterioles contrasts strikingly with their abundance in normally menstruating animals with similar glands and stroma. In such animals, several arteriolar fields, each representing the coils of a single arteriole, are to be found in any section of the entire fundus uteri or an equivalent area of the endometrium. Furthermore, in this stage of proliferative activity the arteriolar fields are large and complex and the vessels show evidence of rapid growth (Kaiser 1947a). In a large number of serial sections of the entire fundus uteri of the two animals studied here, only one poorly developed coiled arteriole was found. It is not possible to state with certainty whether this represents a failure to respond to the hormonal stimulus or a toxic effect of the massive doses employed, since there is no way of knowing the arteriolar patterns of these animals prior to the experiment. The absence of any other evidence of toxicity makes it appear more likely that this represents a failure of the vessels to respond during an excellent response of glands and stroma.

Hormone withdrawal bleeding occurred in the five castrates after an interval of six to nine days, averaging seven days. Rhesus monkeys given estrogens in much smaller amounts usually bleed after an interval of five days. This lag of 2 days may be due to depots of oil left in the animals' legs by a regime of $\frac{1}{2}$ cc. of oil administered intramuscularly each day for 26 days. The occurrence of bleeding in animals given hormone stimulation identical with that of the animal found to have virtually no endometrial coiled arterioles is suggestive evidence that these vessels are not essential for uterine bleeding. Menstruation in the absence of coiled arterioles has been observed in intact New World monkeys (Kaiser 1947b). The significance of its apparent occurrence in castrate rhesus monkeys has been discussed elsewhere.³

Squamous metaplasia of the endocervical epithelium is a common finding in castrated rhesus monkeys treated with estrogens, especially those which have received large doses. In the normal animal there may be an upward extension of the squamous epithelium of the portion vaginalis which simulates squamous metaplasia, but this does not involve the apices of the endocervical glands (Hisaw and Lendrum 1936, Zuckerman 1938). The production of epidermidization has been reported following such low doses of estrogens as 41.5 R.U. a day for 26 days (Hisaw and Lendrum 1936) and even 60 I.U. a day for 21 to 61 days (Migliavacca 1937). It has however been more commonly found after large or massive doses (Overholser and Allen 1933, 1935, Engle and Smith 1935, Zuckerman 1937a, Hartman, Geschickter and Speert 1941, and Vargas 1943). There appears to be some relationship between size of dose and duration of administration

³ Kaiser, I. H.: Newer Concepts of Menstruation. *Am. J. Obs. Gyn.* In press.

for the production of this process, but the series reported are too scattered in regard to dose and duration to allow definite conclusions. It may be reiterated that Overholser and Allen's early suggestion that these changes are precancerous has not been confirmed.

Cystic dilatation of the glands of the endometrium has been observed in rhesus monkeys only after prolonged administration of large doses of estrogens (Zuckerman 1937b, Hartman, Geschickter and Speert 1941, Cleveland, Phelps and Burch 1941 and Vargas 1943). It did not occur in the present experiments despite the use of massive doses, perhaps because of the relatively brief period of administration. Zuckerman and Morse (1935) reported cystic endometrial hyperplasia in a chimpanzee and a mangabey after similarly brief periods, but in the rhesus monkey months of treatment are evidently required for its production.

SUMMARY

Administration of massive doses of estrogens to castrate rhesus monkeys results in withdrawal bleeding of 3 to 7 days' duration after an interval of 6 to 9 days. Microscopic study of the uteri of one castrate and one intact animal, each killed after four weeks of injections, revealed squamous metaplasia of the endocervix but no cystic hyperplasia of the endometrium. There was an excellent proliferative development of endometrial glands and stroma but virtual absence of endometrial coiled arterioles. This dissociation of the growth of these vessels from the growth of the glands and stroma suggests that uterine bleeding can occur in the absence of coiled arterioles in the rhesus monkey as it does in the New World monkeys.

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THE EFFECT OF ALTERED SODIUM OR POTASSIUM INTAKE ON THE WIDTH AND CYTO-CHEMISTRY OF THE ZONA GLOMERULOSA OF THE RAT'S ADRENAL CORTEX¹

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THE cortex of the rat's adrenal gland is divided morphologically into three major zones. The appearance of the cells and the width of the outer two zones, the glomerulosa and fasciculata, can be altered in different physiological states. Thus, the zona fasciculata enlarges and its cells appear active in conditions eliciting an increased production of adrenotropin, which stimulates the release of the cortical "sugar" factors. On the other hand, this zone shrinks and becomes inactive after hypophysectomy. Either change may occur without apparent alteration in the activity of the glomerulosa (Deane and McKibbin, 1946; Deane and Greep, 1946). The present paper will describe experiments indicating that the outer zone, the glomerulosa, undergoes morphological and cytochemical changes in physiological states that involve disturbance of the electrolyte balance of the body. These changes may occur without any demonstrable alteration of the fasciculata. Moreover, they develop in hypophysectomized as well as in intact rats.

The results of two different physiological experiments have led to the hypothesis that, in the rat at least, the zona glomerulosa produces salt-regulating hormones of the 11-desoxycorticosterone type. In the first place, the prolonged administration of desoxycorticosterone acetate causes shrinkage and inactivity of the glomerulosa (Sarasohn, 1943; Greep and Deane, 1947). This observation suggests that disuse atrophy overtakes the zone when its normal product is replaced by the injected hormone. Secondly, adrenalectomized rats lose sodium chloride rapidly through the kidneys, whereas hypophysectomized rats are more nearly normal in this respect. Thus it would seem that the salt-regulating activity of the adrenal is relatively unchanged by

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hypophysectomy. Since the glomerulosa remains intact and apparently active following the operation (Sarason, 1943; Deane and Grep, 1946), it is reasonable to assume that this zone produces salt-retaining hormones of the desoxycorticosterone type and that it is regulated, at least in part, by an extra-pituitary agent.

If the zona glomerulosa in the rat produces desoxycorticosteroids in response to an extra-pituitary stimulus, changes in its activity should accompany situations in which the demand for desoxycorticosteroids was altered, either in intact or hypophysectomized animals. Altered demand for these hormones would be expected to follow changes in the normally high sodium:potassium ratio in the blood stream, since their major function is the maintenance of the normal relation by regulating sodium retention and potassium excretion (Swingle and Remington, 1944). Three deductions have been drawn from this hypothesis.

(1) Increased demand for desoxycorticosteroids should follow a decrease in the sodium:potassium ratio. Two ready means of lowering this ratio exist: (a) When the animal is fed a diet virtually lacking in sodium, blood sodium falls, while blood potassium increases slightly (Turpeinen, 1938); (b) when the animal is injected with large amounts of potassium chloride, blood potassium rises acutely and blood sodium drops somewhat (Miller and Darrow, 1940).

(2) If the sodium:potassium ratio of the blood were elevated, the demand for this type of hormone should be lowered. (a) One method for lowering the level of blood potassium, while leaving blood sodium unaffected, is to feed the animal a diet containing less than 0.01 per cent potassium (Heppel, 1939; Darrow and Miller, 1942). (b) This electrolyte shift may also be accomplished by the administration of desoxycorticosterone acetate, which reduces the potassium level in the blood acutely and raises the sodium level slightly (Darrow and Miller, 1942).

(3) In addition, it might be predicted that the administration of adequate quantities of desoxycorticosterone would protect the animal from a fall in the sodium:potassium ratio produced either by a low sodium diet or by potassium injection, since this hormone promotes sodium retention and potassium excretion by the kidney. If such protection occurred, little or no stimulation to the glomerulosa should follow.

Experiments to test these deductions have been performed on intact and hypophysectomized rats, and the observed changes in the zona glomerulosa of the adrenal cortex are set forth below. A review of the literature has revealed only a few observations on the adrenal cortices of rats whose sodium or potassium intake was abnormal. Sarason (1943) observed no morphological or cytochemical changes in the cortices of rats fed a potassium-deficient diet for a month.

Orent-Keiles, Robinson and McCollum (1937) commented that the adrenal glands appeared hemorrhagic in rats dying after 18-21 weeks on a diet severely deficient in sodium. However, they made no histological examination of this organ.

MATERIAL AND METHODS

The rats used in the following experiments were young males of the Long-Evans strain. They had been raised in our laboratory and fed Purina fox chow after weaning. Before and during the experiments they were supplied with tap water *ad libitum*. Boston water contains 0.3 mg. sodium and 0.2 mg. potassium per 100 cc. (Research Lab., Mead, Johnson).

Experimental Plan

(1) *Rats fed diets deficient in sodium, in potassium, or in sodium and potassium.* To observe the effects of sodium and potassium deficiencies on the glomerulosa, rats weighing approximately 150 grams were fed purified diets deficient in sodium, potassium, or both sodium and potassium. The composition of the basic purified ration has been described previously (Deane and Shaw, 1947), and the various modifications of the salt mixture that were used are listed in Table 1. Six rats were fed the diet deficient in sodium (Diet A), containing only 0.004 per cent sodium.² Six were fed the diet deficient in potassium (Diet B), containing 0.005 per cent potassium. Six were fed the diet deficient in both of these electrolytes (Diet C), containing 0.004 per cent sodium and 0.005 per cent potassium. Six control rats were fed the purified diet containing the usual amounts of sodium and potassium (Diet D). One rat from each of the four groups was killed at 8, 14, 22, 30, 38 and 70 days after the beginning of the experiment.

(2) *Hypophysectomized rats fed diet deficient in sodium.* To determine the rôle of the pituitary in the response of the glomerulosa to sodium deficiency, rats which had been totally hypophysectomized for 2 weeks were transferred to Diet A (Table 1). Controls consisted of operated animals that were fed the complete purified diet (Diet D). The rats weighed approximately 125 grams before hypophysectomy and, at the time of the beginning of the dietary experiment, 100 grams. Two sodium-deficient animals were killed after 2 weeks and three after 4 weeks. One operated animal on the control diet was killed at the end of 4 weeks.

(3) *Rats injected with potassium chloride.* To learn the effects of toxic doses of potassium on glomerulal activity, rats were given intraperitoneal injections of a 5 per cent solution of potassium chloride. The dose selected was 55 mg. per 100 g. body weight, slightly below the MLD determined by Truszkowski and Duszynska (1940) and Emmens and Marks (1942). Ten rats weighing about 125 grams were given a single injection. Two died shortly thereafter. The eight survivors were autopsied at 2, 4, 6 (2 rats), 8, 10, 12 and 18 hours after injection. Two other rats were given two injections

² The sodium and potassium contents of the diets were kindly measured by Dr. Robert E. Olson, Department of Nutrition, Harvard School of Public Health. After wet digestion of the mixture, determinations were made on a Perkin-Elmer flame photometer with an internal lithium standard.

tions of 55 mg. per cent 12 hours apart, and the one survivor was killed 12 hours after the second injection.

To learn whether the repeated administration of smaller and less toxic doses of potassium chloride would affect the adrenal, three rats were injected four times with 20 mg. per 100 g. at 2-hour intervals. One rat died immediately after the fourth injection (6 hours) and was autopsied. The other two were killed 24 hours after the first injection.

(4) *Rats fed diet lacking sodium and injected with desoxycorticosterone acetate.* To determine whether the administration of desoxycorticosterone would protect the adrenal cortex from the effects of sodium deficiency, four young rats weighing about 50 grams were fed Diet A (Table 1) and

TABLE 1. COMPOSITION OF DIETS

<i>Basal diet</i>		<i>g.</i>
Sucrose		670
Casein (SMA)		240
Corn oil		50
Vitamin mixture ^{a,b}		
Basal salt mixture		30
<i>Basal salt mixture for ration</i>		<i>g.</i>
Mono-calcium ortho-phosphate		980.0
Calcium chloride, anhydrous		570.0
Magnesium sulfate, anhydrous		100.0
Ferrie citrate		55.0
Manganous sulfate, anhydrous		9.0
Zinc chloride, anhydrous		4.0
Cupric sulfate, anhydrous		2.4
Potassium iodide		1.6
Cobaltous sulfate		0.2
<i>Additional salts in the four diets</i>		
<i>Diet A (sodium-deficient)</i>		
Potassium carbonate		8.0 g.
<i>Diet B (potassium-deficient)</i>		
Sodium carbonate		10.4 g.
<i>Diet C (sodium and potassium-deficient).</i>		
nothing		
<i>Diet D (complete)</i>		
Potassium carbonate		8.0 g.
Sodium carbonate		10.4 g.

^a Deane and Shaw (1947)

^b We are indebted to Merck and Co., Inc., Rahway, N. J., for the crystalline vitamins used in these diets.

injected daily with 2 mg. desoxycorticosterone acetate in sesame oil (Percorten, Ciba³). This dose produces involution of the glomerulosa in animals receiving a normal diet (Grep and Deane, 1947). Two of these rats were killed after 2 weeks of treatment and two after 4 weeks. As controls, two rats were fed Diet A only, and two rats receiving 2 mg. desoxycorticosterone daily were fed the complete diet (Diet D). One control of each type was killed at 2 weeks and at 4 weeks after beginning the experiment.

Additional control animals for the last three experiments consisted of intact rats fed Purina fox chow.

³ The Percorten was generously supplied by the Ciba Pharmaceutical Products, Inc., Summit, N. J., through the courtesy of Dr. E. Oppenheimer.

Histological Procedures

All of the rats were killed by a blow on the head. The paired adrenal glands and the thymus gland were carefully dissected and weighed on a Roller-Smith torsion balance. In the last three experiments the paired kidneys were weighed as well, since an increase in kidney size with potassium deficiency had been noted in the first one.

One adrenal from each rat was fixed in 10 per cent neutralized formalin. After fixation for at least 48 hours, it was washed in running tap water for an hour, then sectioned on the freezing microtome at 15μ . One section was stained with sudan IV and Harris' hematoxylin, another with sudan black B and a third by the Schiff plasmal method; these were all mounted in glycerin jelly. Two additional sections were mounted unstained in glycerin—one was untreated, and the other was extracted with acetone at room temperature for one-half hour. The latter two sections were compared under the polarizing and the fluorescence microscopes for the presence of acetone-soluble substances showing birefringence and greenish-white autofluorescence. Any acetone-soluble, sudanophilic droplet that was also Schiff-positive, autofluorescent and birefringent was assumed to contain ketosteroids, since no other single substance is known to react positively to all of these tests (Dempsey and Wislocki, 1946). These reactions presumably occur in the sites of formation of the adrenal hormones, which fall into the class of ketosteroids.

Variations in the width of the zona glomerulosa were noted following the various experimental procedures. Measurements of this zone were made on photomicrographs of the sections. These measurements have been converted to micra and are incorporated in the descriptions. Since the variations are readily detectable in the illustrations and were consistent among the animals in each group, no attempt was made to take sufficient measurements to treat them statistically. Rather, these data are included to permit ready comparison of zone-width following the various experimental procedures.

RESULTS

1. *Intact rats fed diets deficient in sodium, in potassium, or in sodium and potassium.* The rats fed the complete purified diet (Diet D) gained weight steadily, whereas the rats fed the diets deficient in either sodium (Diet A) or potassium (Diet B) gained weight slowly, and those fed the diet deficient in both elements (Diet C) lost weight for the first 3 weeks (Fig. 1). Table 2 presents the data concerning the average weight changes for all the rats on each of the four diets.

The average proportional weights of the adrenal glands and thymuses of the six animals in each group are also listed in Table 2. The proportional weights of the adrenals and thymuses of the control animals were normal for rats of this age and size. The adrenal weights of the rats on the various deficient diets were slightly higher, but their thymus weights were within the normal range. The kidneys were not weighed in this experiment, but it was noted that those of the potassium-deficient rats appeared pale and considerably enlarged, as described by Follis, Orent-Keiles and McCollum (1942) and Dur-

TABLE 2. AVERAGE WEIGHT DATA FOR INTACT RATS ON DIETS DEFICIENT IN SODIUM, POTASSIUM, AND SODIUM-AND-POTASSIUM, AND FOR THEIR CONTROLS.
INDIVIDUAL RATS FROM EACH GROUP WERE KILLED AT
8, 14, 22, 30, 38 AND 70 DAYS

Diet	No. rats	Initial weight g.	Final weight g.	Change in weight g.	Adrenals	Thymus
					(Proportion, mg./100 g. body weight)	
Complete (D)	6	153	222	+69	17.0	176
Na-deficient (A)	6	128	165	+37	22.8	218
K-deficient (B)	6	143	162	+19	22.9	187
Na, K-deficient (C)	6	140	126	-14	24.7	168

lacher, Darrow and Winternitz (1942). The kidneys of the rats on the other diets seemed normal in size and appearance.

The adrenal cortices of the rats on the complete purified diet (D) appeared normal in the cytochemical preparations. The zona glomerulosa was somewhat irregular and measured 30–40 μ in width. Its cells were quite small and generally crowded with sudanophilic droplets. The lipid content of different parts of the zone showed the normal variability. The sudanophilic droplets contained considerable

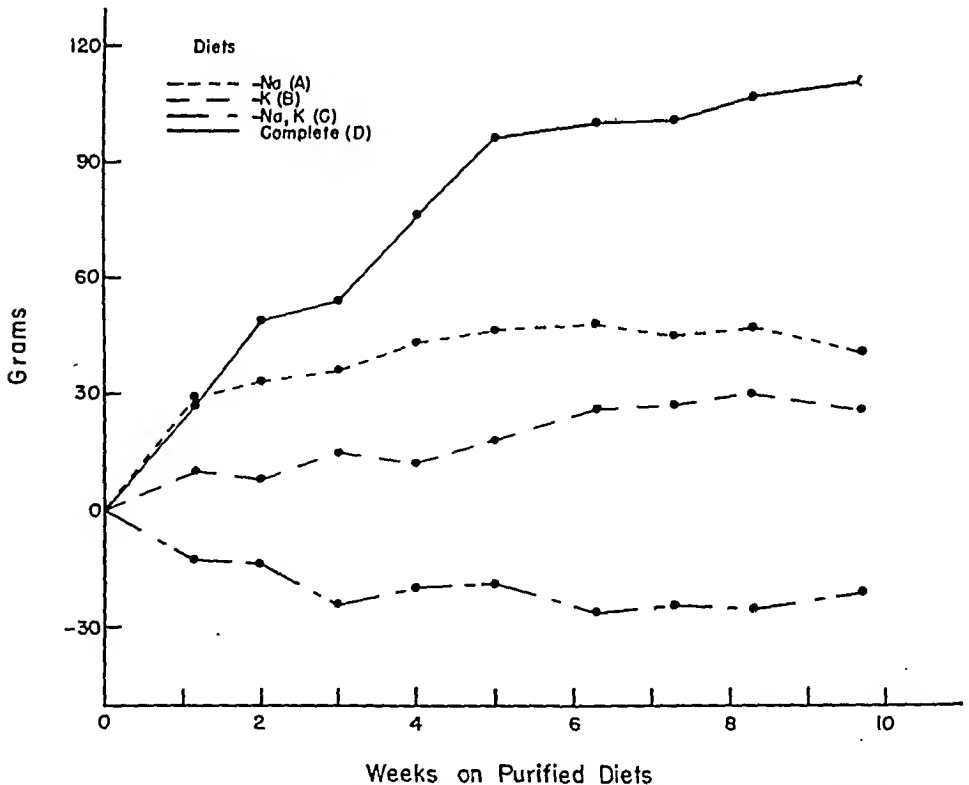


FIG. 1. The change in body weight over a period of 70 days of individual rats on the different purified diets. Initially the animals weighed approximately 150 grams. The weight changes of these animals were representative for their respective groups.

ketosteroid as evidenced by the cytochemical criteria employed (Figs. 3, 8 and 12). The birefringent particles were mixed in size. Underneath the glomerulosa lay a narrow "transitional" zone possessing few lipid droplets. The broad zona fasciculata contained numerous ketosteroid droplets in its outer part and fewer in its inner part. Little or no zona reticularis was present in the cortices of these young rats. The occasional fatty cells in this zone failed to display the ketosteroid reactions and therefore probably contained only triglycerides.

In the animals fed the sodium-deficient diet, the glomerulosa became conspicuously broader than normal, measuring 60μ by the eighth day (Fig. 4). On high-power examination, cell hypertrophy was seen to account for this increase in width of the zone. The lipid droplets in the cells were smaller, sparser, less sudanophilic and more irregular in distribution than usual, and the birefringent particles were uniformly fine. By 22 days the zone had become still broader and further depleted of lipid. Furthermore, the cells had developed large watery vacuoles (Figs. 5, 18 and 20) like those which characterize the cells of the outer fasciculata during the extreme depletion attending the adaptation syndrome (Deane and Shaw, 1947, Fig. 2). After 38 to 70 days, the glomerulosa measured 70 to 100μ and was almost completely depleted of all material which was sudanophilic, Schiff-positive, autofluorescent and birefringent (Figs. 5, 9, 13). No particular changes were noted in the zona fasciculata of the rats fed the sodium-deficient diet (Fig. 13).

In the potassium-deficient rats, the zona glomerulosa became somewhat narrower than normal (20μ) and the cells smaller. The sudanophilic droplets became enlarged and compactly arranged (Fig. 6), while the Schiff reaction (Fig. 10) and autofluorescence of the droplets gradually underwent a diminution. Furthermore, the birefringent particles all became coarse in character (Fig. 14). In the fasciculata, the ketosteroid reactions seemed slightly enhanced, and the birefringent particles appeared crowded and relatively fine (Fig. 14).

In rats fed the diet deficient in both sodium and potassium, the zona glomerulosa was noticeably broadened by the 22nd day, attaining a width of 60μ by the 70th day. The individual cells were detectably enlarged. Fine lipid droplets, which were intensely sudanophilic (Fig. 7), Schiff-positive (Fig. 11), and autofluorescent, persisted in this zone throughout the course of the experiment. The birefringent particles appeared uniformly fine and densely crowded (Fig. 15). On this diet, the zona fasciculata appeared essentially normal (Fig. 15).
2. *Hypophysectomized rats fed diet deficient in sodium.* The hypophysectomized rats were maintained for 2 weeks on fox chow in order to permit recovery from post-operative depletion of the glomerulosa (Deane and Greep, 1946). Some were then fed the diet lacking sodium (A) and others the complete diet (D). Only a small number of

TABLE 3. AVERAGE WEIGHT DATA FOR RATS HYPOPHYSECTOMIZED AND FED FOX CHOW FOR TWO WEEKS, THEN FED THE SODIUM-DEFICIENT (A) OR COMPLETE (D) PURIFIED DIETS

	No. rats	Operated, wks.	Diet, wks.	Wt. g.	Adrenals	Thymus	Kidneys
					(Proportion, mg./100 g. body wt.)		
Intact	2	-	Chow	101	20.6	240	1090
Hypophysectomy only	1	6	(Chow, 2) D, 4	99	10.9	146	687
Hypophysectomy, -Na	2	4	(Chow, 2) A, 2	101	9.7	154	778
Hypophysectomy, -Na	3	6	(Chow, 2) A, 4	101	8.1	62	738

operated animals survived for the 6 weeks required for the experiment.

The adrenal, thymus and kidney weights for hypophysectomized rats fed the sodium-deficient diet (A) are compared in Table 3 with

EXPLANATION OF FIGURES ON PLATE I

All photomicrographs on this and the succeeding plates are of adrenal glands which were fixed in 10 per cent neutralized formalin for over 48 hours, washed thoroughly, and sectioned at 15μ on the freezing microtome.

Figs. 3-11. Exp. 1. The glomerulosa and outer part of the fasciculata of the adrenal cortex. The border between the glomerulosa and the transitional zone has been drawn in. Figs. 3-7, Sudan IV and hematoxylin, photographed with blue filter. Figs. 8-11, Schiff plasmal reaction, photographed with combined green and yellow filters. All figures $\times 200$.

FIG. 3. Rat fed complete purified diet (D, Table 1) for 70 days. The zona glomerulosa is characteristically irregular in width and moderately narrow (30μ). The sudanophilia of the lipid droplets in this zone is intense. Beneath the glomerulosa are a few rows of cells which contain little or no lipid—the "transitional" zone. The cells of the outer part of the zona fasciculata are well filled with sudanophilic droplets.

FIG. 4. Rat fed the sodium-deficient diet (A) for 8 days. The glomerulosa is broadened (60μ) and its cells enlarged. They contain numerous fine lipid droplets which are less intensely sudanophilic than normal. Watery vacuoles have developed in some of the cells.

FIG. 5. Rat fed the sodium-deficient diet (A) for 38 days. The glomerulosa is further broadened (75μ) and retains only a few fine sudanophilic droplets. The cells exhibit numerous large watery vacuoles.

FIG. 6. Rat fed potassium-deficient diet (B) for 70 days. The glomerulosa appears shrunken (20μ), but filled with intensely sudanophilic droplets.

FIG. 7. Rat fed purified diet lacking both sodium and potassium (C) for 38 days. The glomerulosa is broad (55μ), the cells enlarged and crowded with markedly sudanophilic droplets.

FIG. 8. Rat fed complete purified diet (D) for 38 days. The Schiff reaction parallels the sudanophilia in the glomerulosa and outer fasciculata in the normal gland. The glomerulosa measures 40μ .

FIG. 9. Rat fed sodium-deficient purified diet (A) for 38 days. Only a few Schiff-positive droplets remain in the broad glomerulosa (100μ), although the reaction in these few droplets remains intense.

FIG. 10. Rat fed potassium-deficient diet (B) for 38 days. Not only is the glomerulosa narrower than normal (20μ), but the droplets possessing Schiff-positive material are sparse and give only a weak reaction.

FIG. 11. Rat fed purified diet lacking both sodium and potassium (C) for 38 days. The abundant lipid droplets in the broad glomerulosa (50μ) are intensely Schiff positive.

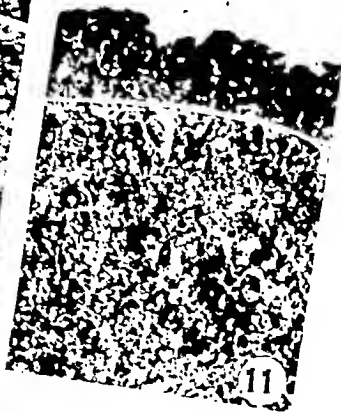
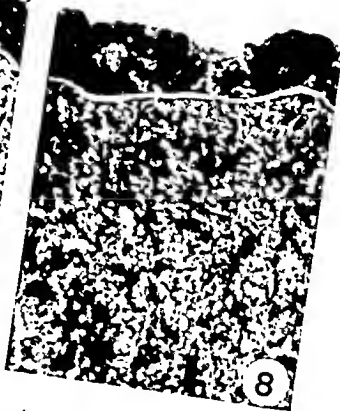
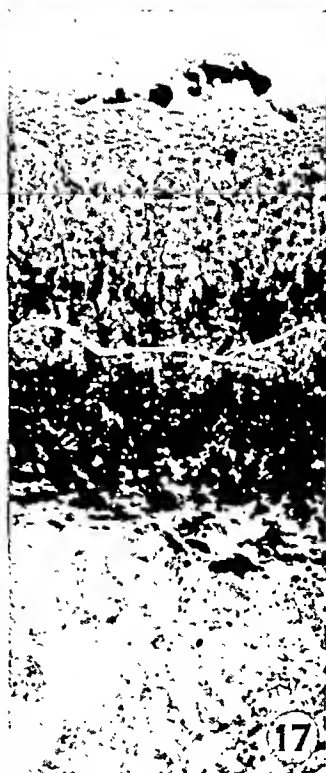
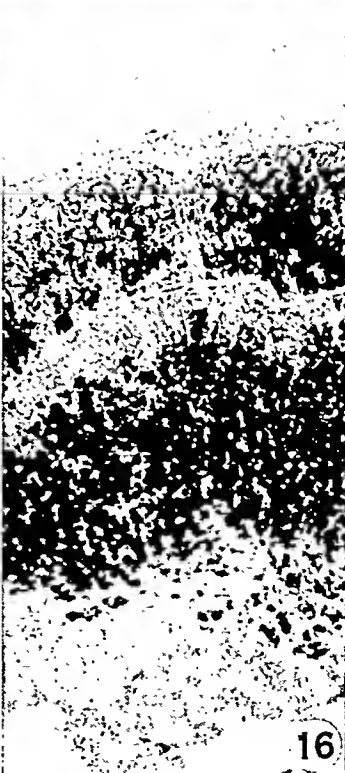
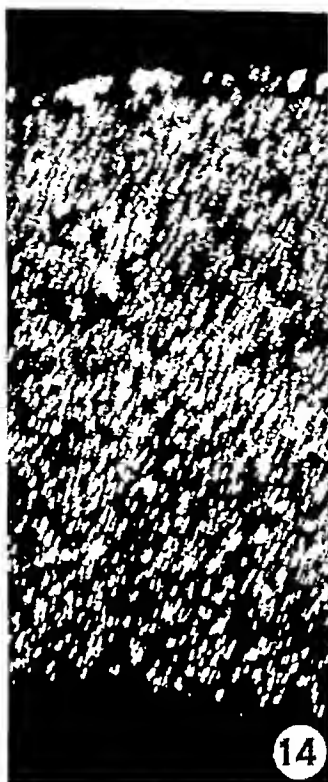
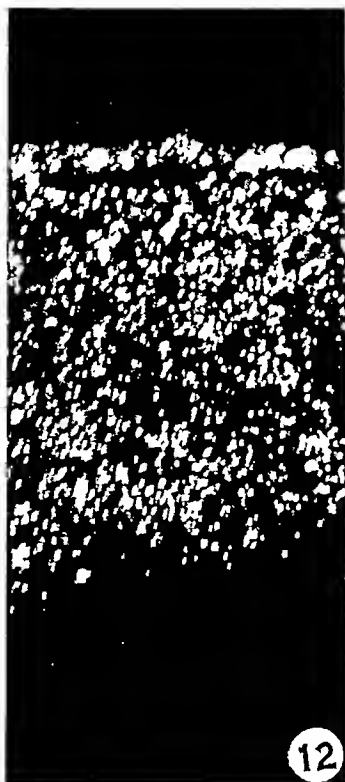


PLATE I



those for an operated rat fed the complete diet (D) and with those for intact rats of the same weight. The adrenals (Deane and Greep, 1946), thymus (Smith, 1930), and kidneys (McQueen and Thompson, 1940; Selye, 1941) regressed in all the hypophysectomized rats. However, the kidneys from the animals receiving the sodium-deficient diet were slightly heavier than those from the rats fed the complete diet.

Histologically the adrenal cortex of the operated animal that was fed the complete purified diet showed an over-all atrophy similar to that in glands of animals maintained on chow for 6 weeks after the operation. The latter have been described in considerable detail by Deane and Greep (1946) and Greep and Deane (1947). The over-all atrophy resulted from marked shrinkage of the fasciculata and reticularis, whereas the glomerulosa was broader than normal (120μ) (Fig. 16). In the glomerulosa all of the ketosteroid reactions remained intense, but in the shrunken inner cortex the residual large sudanophilic droplets were neither Schiff-positive nor autofluorescent and were only occasionally birefringent. Consequently, the droplets remaining in the inner zone probably contained triglycerides without any ketosteroids dissolved therein.

In contrast to the control hypophysectomized rat, the rats fed the sodium-deficient diet exhibited a progressive depletion of ketosteroids in the zona glomerulosa. By the fourth week, lipid droplets displaying the various reactions were completely absent from the outer part of

EXPLANATION OF FIGURES ON PLATE II

FIGS. 12-15. Exp. 1. Birefringence pattern in unstained sections of the whole adrenal cortex. $\times 100$.

FIG. 12. Rat fed complete purified diet (D) for 70 days. A mixture of fine and coarse birefringent particles appears in the narrow glomerulosa (30μ) and in the fasciculata. The "transitional" zone between them is empty.

FIG. 13. Rat fed sodium-deficient diet (A) for 38 days. The birefringent capsule outlines the gland. Below this the glomerulosa (70μ) is completely devoid of birefringent material. The fasciculata presents about the usual amount.

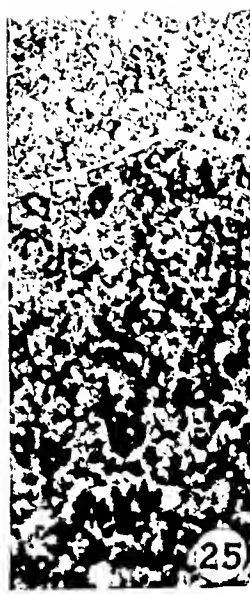
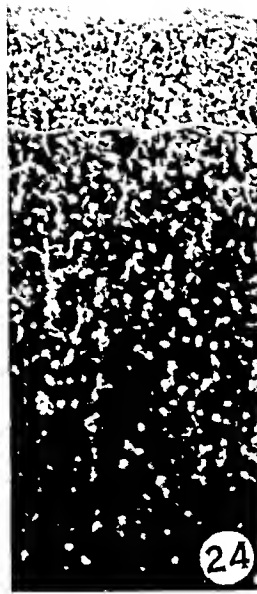
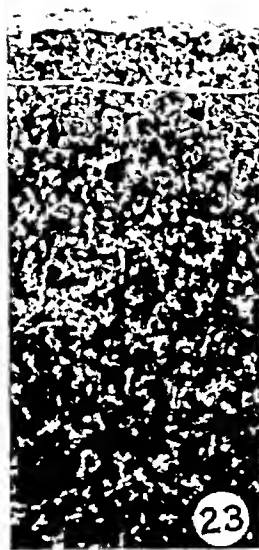
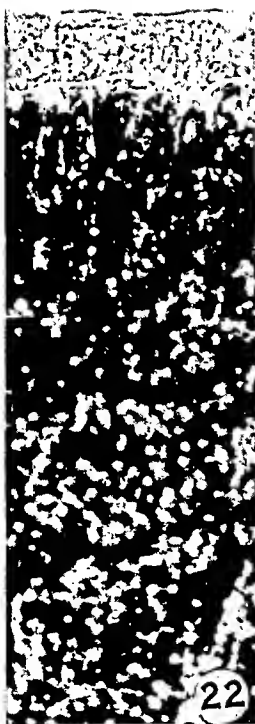
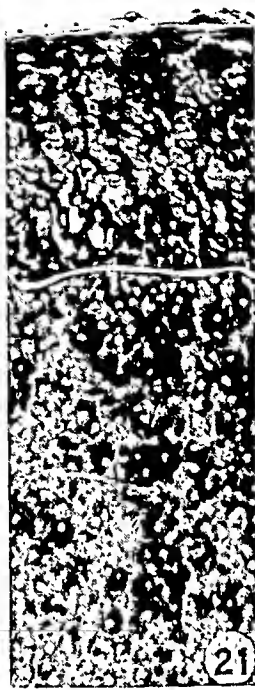
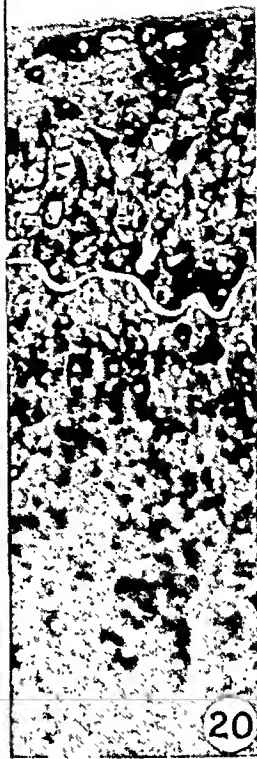
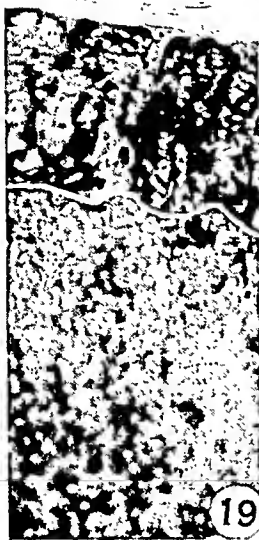
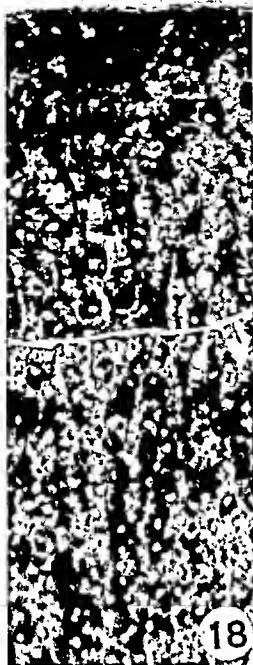
FIG. 14. Rat fed potassium-deficient diet (B) for 70 days. The glomerulosa (20μ) displays only coarse particles. Moreover, the crystals appear sparser than normal (Fig. 12). The fasciculata seems somewhat superactive, displaying numerous fine birefringent particles.

FIG. 15. Rat fed purified diet lacking both sodium and potassium (C) for 70 days. The broad glomerulosa (60μ) appears crowded with small birefringent particles. The fasciculata contains particles of both sizes as usual.

FIGS. 16 and 17. Exp. 2. Adrenal cortices from rats hypophysectomized for 6 weeks. Line drawn between the glomerulosa and "transitional" zone. Sudan IV and hematoxylin, photographed with a blue filter. $\times 150$.

FIG. 16. Control rat hypophysectomized for 6 weeks and fed complete purified diet (D) for the last 4 weeks. Sudanophilic lipids occur in the broad glomerulosa (120μ) and in the shrunken inner zones. By comparison with the other methods, it appears that only the droplets in the glomerulosa contain ketosteroids.

FIG. 17. Rat hypophysectomized 6 weeks and fed sodium-deficient purified diet (A) for 4 weeks. The glomerulosa is considerably broader (160μ) than in Fig. 16, and is almost completely depleted of lipids, which persist only at the innermost border of the zone. The residual lipid droplets in the glomerulosa display the various ketosteroid reactions.



the glomerulosa, although a few remained in the cells at the inner border of the zone (Fig. 17). Moreover, the glomerulosa became broader than in the control gland ($160/120\mu$), as in intact animals receiving the sodium-deficient diet.

3. *Intact rats injected with potassium chloride.* A single intraperitoneal injection of 55 mg. potassium chloride per 100 g. body weight proved fatal in two out of ten rats. Cytochemical preparations of the adrenals of the survivors demonstrated a considerable depletion of ketosteroids from the zona glomerulosa as early as 4 hours after injection (Fig. 23). This reduction progressed until 12 hours (Fig. 24), when any lipid droplets that persisted were small. By 12 hours the zone had broadened (55μ) and the cells were enlarged. By 18 hours the zone was partially restored to normal.

In the rats receiving four injections of smaller quantities of potassium chloride (20 mg. per 100 g.), the glomerulosa became extremely broad (85μ) and almost completely depleted of ketosteroids by 6 hours (Fig. 25). Almost complete restoration had occurred by 24 hours. In no case did the fasciculata appear significantly changed following potassium injection.

4. *Intact rats fed diet deficient in sodium and injected with desoxycorticosterone acetate.* To ascertain whether the concomitant adminis-

EXPLANATION OF FIGURES ON PLATE III

Figs. 18-22. Exp. 4. Glomerulosa and outer fasciculata; line drawn between the glomerulosa and "transitional" zone. Sudan black B, photographed with a blue filter. $\times 200$.

Fig. 18. Rat fed sodium-deficient purified diet (A) for 2 weeks. The glomerulosa is conspicuously broadened (175μ) but still contains considerable sudanophilic material. Note watery vacuoles in the cells.

Fig. 19. Rat fed sodium-deficient diet (A) and injected with desoxycorticosterone acetate for 2 weeks. The glomerulosa (105μ) is less enlarged than in Fig. 18 but contains about the same amount of lipid.

Fig. 20. Rat fed sodium-deficient diet for 2 weeks. Glomerulosa broad (150μ) and almost completely depleted of lipid. Conspicuous watery vacuoles.

Fig. 21. Rat fed sodium-deficient diet and injected with desoxycorticosterone acetate for 4 weeks. Glomerulosa (140μ) nearly as broad as in Fig. 20 and, if anything, more depleted of lipid.

Fig. 22. Rat fed complete purified diet (D) and injected with desoxycorticosterone acetate for 4 weeks. Glomerulosa narrow (25μ) and completely depleted of lipids. Contrast with Figs. 19 and 21. Fasciculata densely packed with lipid.

Figs. 23-25. Exp. 3. Rats injected with potassium chloride. Glomerulosa and outer fasciculata; line drawn between glomerulosa and "transitional" zone. Sudan IV and hematoxylin, photographed with blue filter. $\times 200$.

Fig. 23. Rat injected 4 hours previously with 55 mg. potassium chloride per 100 g. body weight. The glomerulosa remains at normal width (30μ) but is conspicuously depleted of lipid droplets. Those remaining are exceedingly small.

Fig. 24. Rat injected 12 hours previously with 55 mg. potassium chloride per 100 g. body weight. The glomerulosa now appears considerably broadened (55μ) and its cell enlarged. The zone is further depleted of lipid droplets.

Fig. 25. Rat injected 4 times at 2-hour intervals with 20 mg. potassium chloride per 100 g. body weight. Died after fourth injection (6 hours). The glomerulosa is very broad (85μ) and its cells conspicuously enlarged. The zone appears almost completely depleted of lipid droplets.

tration of desoxycorticosterone acetate would prevent the changes in the glomerulosa resulting from sodium deficiency, rats weighing 50 grams were placed on Diet A and injected with 2 mg. hormone daily for as long as a month. Controls consisted of rats fed the deficient diet only or injected with hormone only.

The weights at autopsy of the paired adrenal glands, the thymuses and the paired kidneys of the experimental animals and their dietary and hormone-injected controls are recorded in Table 4. The control rats injected with desoxycorticosterone acetate showed adrenal

TABLE 4. AVERAGE WEIGHT DATA FOR THE RATS FED A SODIUM-DEFICIENT DIET (A) AND INJECTED DAILY WITH 2 MG. DESOXYCORTICOSTERONE ACETATE, AND FOR THEIR CONTROLS

Diet	No. rats	Hormone treatment	Initial wt., g.	Final wt., g.	Wt. gain, g.	Adrenals	Thymus	Kidneys
						(Proportion, mg./100 g. body wt.)		
Chow	2	—	—	84	—	22.8	242	1185
Na-deficient (A), 2 wks.	1	—	61	90	+29	31.2	186	1170
Na-deficient (A), 2 wks.	2	+	57	80	+22	28.6	243	1185
Complete (D), 2 wks.	1	+	47	76	+29	20.9	298	1270
Na-deficient (A), 4 wks.	1	—	56	82	+26	30.2	200	1190
Na-deficient (A), 4 wks.	2	+	53	83	+30	26.4	229	1190
Complete (D), 4 wks.	1	+	44	143	+99	17.5	250	1330

atrophy, a result which has been attributed to inhibition of the secretion of adrenotropin from the pituitary by this hormone (Del Castillo and Rapela, 1945; Grep and Deane, 1947). The kidneys hypertrophied, probably because of potassium deficiency (Durlacher, Darrow and Winternitz, 1942). On the other hand, those control rats fed the diet lacking sodium showed a slight enlargement of the adrenals but no alteration in kidney weight. Finally, the experimental animals given both treatments assumed an intermediate position between the other two groups in respect to adrenal size, but their kidney weights were normal. This latter fact might indicate that low blood sodium tends to offset the effect of low blood potassium on kidney size. The thymuses of all three groups of animals were within the normal range.

The adrenal cortices of the hormone-injected controls resembled those described previously by Grep and Deane (1947), in that the zona glomerulosa atrophied somewhat (25 μ) and its cells shrank (Fig. 22). At 2 weeks the cells of the glomerulosa contained a few large lipid droplets which failed to autofluoresce or react with the Schiff reagent; the birefringent particles were coarse and sparse. By

the end of 4 weeks no lipid material whatsoever remained. The fasciculata also appeared somewhat shrunken, its cells displaying enlarged ketosteroid droplets. The earlier experiments (Greep and Deane, 1947) showed that the glomerulosa of hypophysectomized rats undergoes similar involution with the injection of desoxycorticosterone.

The adrenals of the dietary controls resembled those described in the first experiment (Diet A). However, the zona glomerulosa was even more enlarged in these small rats (av. 163μ , Figs. 18 and 20) than in the larger animals used before. Lipid droplets had almost completely disappeared from the zone by 4 weeks (Fig. 20).

When desoxycorticosterone acetate injection was combined with the sodium-free diet, the effects were similar to those of simple sodium deficiency. The glomerulosa became progressively broader (av. 123μ) and depleted of ketosteroid droplets (Figs. 19 and 21). The only difference between these hormone-treated animals fed the deficient diet and the dietary controls lay in a slightly slower enlargement of the glomerulosa ($105/175\mu$ at 2 weeks; $140/150\mu$ at 4 weeks).

DISCUSSION

In the hypothesis on which these experiments were based it was proposed that, in the rat, the zona glomerulosa forms desoxycorticosterones and that stimulus to their secretion does not require the presence of the anterior pituitary gland. The desoxycorticosterones act, in part, to maintain a high sodium: potassium ratio in the blood plasma. Consequently, it was deduced that any alteration effected in this ratio might alter the demand for the hormones in the attempt of the animal to restore electrolyte equilibrium. In the present experiments the normal ratio was either raised or lowered in intact and hypophysectomized rats, resulting in marked changes in the size and cytochemical characteristics of the zona glomerulosa.

Interpretation of secretory changes in the morphological and cytochemical changes noted in the glomerulosa must depend for the present on analogy with secretory changes in other glandular tissues. The principal metabolic tests developed for desoxycorticosteroids require measurement of blood and urinary electrolytes to determine deviations from normal (Swingle and Remington, 1944). Since the electrolyte levels of the animals used in these studies had been altered by the experimental procedures, we had no metabolic method for evaluating the amount of hormone secreted. However, an interpretation based on analogy with similar changes in the cells of the zona fasciculata should be possible, since the cells have a common embryologic origin and secrete similar products.

A number of workers have correlated the morphological and histochemical alterations in the fasciculata with functional tests for 11-oxygenated corticosteroids. By so doing it has been possible to ascer-

tain the signs that accompany increased or decreased secretory activity.

During increased secretory activity (resulting from pituitary stimulation), the cells of the fasciculata enlarge and, consequently, the zone broadens. The lipid droplets become small but retain their autofluorescence and continue to react intensely with the Schiff reagent. All of the birefringent particles within the droplets become fine (Weaver and Nelson, 1943). Ketosteroid droplets may disappear entirely from the cells if stimulation is excessive or prolonged (Dalton *et al.*, 1944; Deane and Shaw, 1947; Sayers and Sayers, 1948).

On the other hand, with decreased activity of the fasciculata (resulting from reduced pituitary activity), the zone and its cells shrink measurably. In this condition the lipid droplets enlarge and the birefringent particles become coarse. Secondly, the autofluorescence and reactivity with the Schiff reagent decline. With complete inactivity following hypophysectomy, the droplets ultimately disappear entirely (Sarason, 1943; Deane and Grep, 1946; Grep and Deane, 1947). From this fact it can be seen that disappearance of droplets *per se* fails to indicate whether the cell is hyperactive or entirely inactive. Cell size is the primary consideration in evaluating which condition obtains.

Fig. 2 summarizes in diagrammatic form these conclusions concerning fasciculata-cell changes with stimulation or inactivation. It seems reasonable to extend this interpretation of cell activity which has been evolved for the zona fasciculata to the observed changes in the zona glomerulosa.

Hyperactivity of the zona glomerulosa. In the present experiments, whenever a reduction occurred in the usual sodium:potassium ratio of the blood, the alterations characterizing the glomerulosa indicated hyperactivity. Similar changes occurred whether the reduction in ratio was produced by potassium injection (Exp. 3) or by feeding a low-sodium diet (Exp. 1A). Moreover, similar alterations appeared in hypophysectomized rats that were made sodium-deficient (Exp. 2). The glomerulosa broadened and the individual cells hypertrophied. Furthermore the ketosteroid droplets within the cells became very small, while retaining their autofluorescence and reactivity with the Schiff reagent. Their birefringence became uniformly fine. With severe potassium intoxication or prolonged sodium deficiency, the droplets disappeared entirely. The acute depletion with potassium poisoning resembled the depletion of the fasciculata in the "alarm reaction" of Selye (1946) (Fig. 2B). The depletion attending prolonged sodium deficiency followed a stage in which the cells were filled with tiny droplets (Fig. 2C). This depletion therefore appeared to follow the course A→C→D (Fig. 2). With either treatment, extreme depletion probably indicated that the cells were releasing hormone as fast as it

ADRENAL CORTICAL CELLS

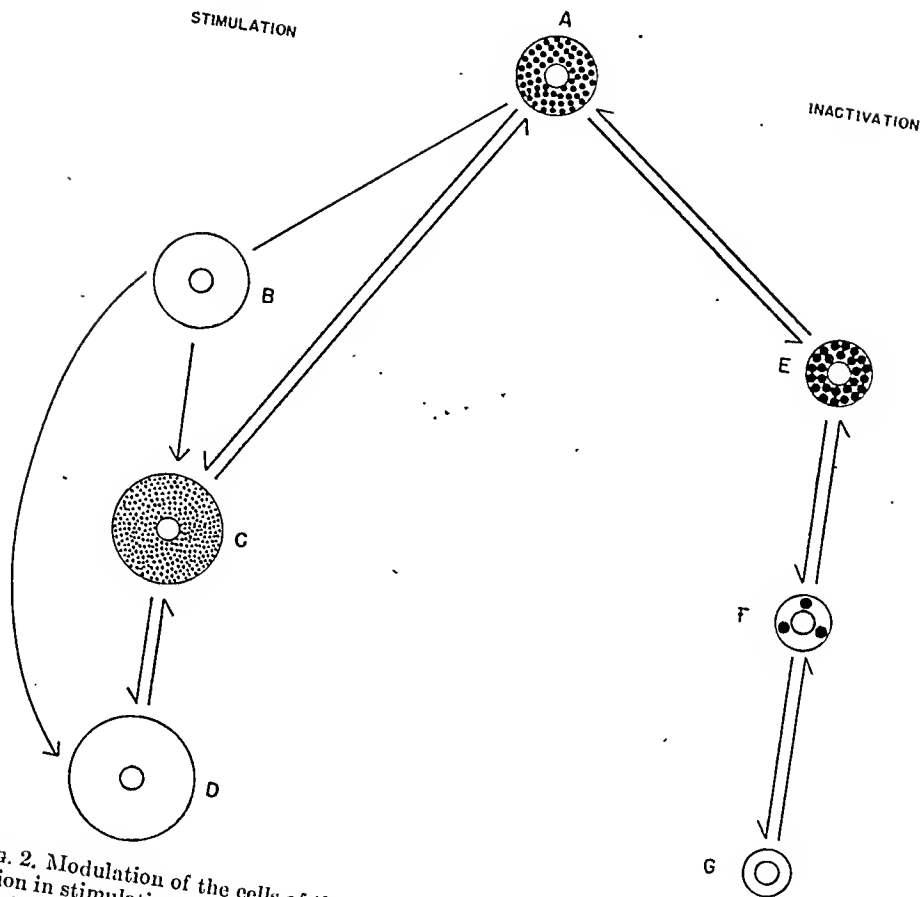


FIG. 2. Modulation of the cells of the fasciculata with increased stimulation or with reduction in stimulation (inactivation). The morphological changes in the glomerulosa observed in the present experiments are interpreted similarly.

A. Normal cell containing moderate sized ketosteroid droplets.
 B, C, D. The cells enlarge following stimulation to increased activity. Ketosteroid droplets when present are unusually small. The course of modulation depends on the acuteness and degree of stimulation. With the most severe stimulus, the course will be $A \rightarrow B \rightarrow D$; with more moderate stimulus, $A \rightarrow B \rightarrow C \rightarrow D$; with mild stimulation, $A \rightarrow C$. Reduction in the stimulus permits return of the cells toward normal along the pathway indicated.
 E, F, G. The cells shrink with loss or reduction of normal stimulation. The ketosteroid droplets enlarge and gradually diminish in number. The extent of modulation depends both on the degree of loss of stimulus and on the duration of inactivation. Reversal occurs when stimulus is reestablished.
 It should be noted that disappearance of lipid droplets may occur either with increased stimulation or with inactivation. Cell size must always be taken into account when judging the activity of the depleted cell.

was manufactured, so that no intracellular storage could occur. Secretory activity had therefore reached its peak.

Consequently it may be concluded that a lowered sodium:potassium ratio elicits an increased secretion of desoxycorticosteroids. This stimulation does not require the presence of the pituitary gland. However, the nature of the stimulating agent awaits further experimentation. Perhaps the reduced electrolyte ratio *per se* causes glomerulosa activation, or perhaps some entirely unknown humoral change stimulates the zone.

Similar but less extreme activation of the glomerulosa occurred when rats were fed a diet lacking both sodium and potassium (Exp. 1C). The zone became considerably broadened, and the ketosteroid droplets within the cells became uniformly fine but, nevertheless, densely crowded throughout the experimental period of 10 weeks. Thus, under the conditions of this experiment stimulation was not severe enough to cause depletion (Fig. 2C). No reports exist in the literature on the electrolyte levels in animals fed such a diet, nor have we made any direct observation on this point. Nevertheless, the reaction of the glomerulosa would indicate that the ratio of sodium and potassium had been lowered somewhat, although not to the degree produced by sodium deficiency alone.

Decreased activity of the zona glomerulosa. In contrast to the preceding situation, whenever the sodium:potassium ratio in the blood stream was elevated, the glomerulosa changes indicated loss of stimulation and inactivity. Extreme inactivation resulted when 2 mg. desoxycorticosterone acetate was injected every day for a month (Exp. 4). The glomerulosa cells shrank. The ketosteroid droplets enlarged and their birefringence became uniformly coarse. They gradually lost their autofluorescence and ability to react with the Schiff reagent; their numbers declined, so that by the end of a month all of them had disappeared (Fig. 2G). Similar changes have been described in hypophysectomized rats injected with desoxycorticosterone (Grep and Deane, 1947). Therefore, neither release from activity nor stimulation requires the presence of the pituitary.

Less marked inactivation of the glomerulosa occurred when the rats were fed a diet deficient in potassium (Exp. 1B). While the glomerulosa shrank and the ketosteroid reactions faded, some droplets persisted within the cells for as long as 10 weeks (Fig. 2F). Almost the same alterations in sodium and potassium levels occur with desoxycorticosterone administration and potassium deficiency (Darrow and Miller, 1942). Consequently the difference in degree of reaction by the glomerulosa would seem to depend on other factors. Since desoxycorticosterone affects functions which potassium deficiency does not (e.g., water balance [Gaunt, 1944] and urea excretion [Ferrebee *et al.*, 1941]) differences in these functions may account for the different results of the two treatments.

Sarason (1943) failed to observe signs of glomerulosa inactivity in rats fed a potassium-deficient diet for a month. Since, in our experience, the glomerulosa is depressed but never becomes entirely depleted with this treatment, his failure points up the necessity for using all of the ketosteroid methods, especially the Schiff reaction, in order to reveal evidences of mild secretory inactivity.

Sarason also emphasized this discrepancy but not with a low-potassium diet. He attributed this discrepancy to fundamentally different effects of the two treatments on the cortex. Since our observations indicate that the two treatments both result in glomerulosa shrinkage and hypoactivity, however, it must have another basis. The administration of desoxycorticosterone is now believed to cause some inhibition of adrenotropin release from the pituitary, an effect variously possessed by all of the cortical hormones (Sayers and Sayers, 1947). This lowered adrenotropin output, in turn, would allow some shrinkage of the fasciculata (Greep and Deane, 1947). Since it has never been found that potassium deficiency inhibits the pituitary, the difference observed by Sarason in the effect of the two treatments on adrenal size may probably be explained on this basis.

The administration of desoxycorticosterone to sodium-deficient rats. Treatment with 2 mg. desoxycorticosterone acetate daily failed to mitigate the stimulus of sodium deficiency to any significant degree. The glomerulosa became nearly as broad and just as depleted in young rats receiving hormone as in rats fed the deficient diet alone. Perhaps the slower enlargement of the zone with hormone treatment indicated some benefit. Nevertheless the additional desoxycorticosterone was apparently incapable of preventing for long a normal sodium:potassium ratio. Two explanations appear completely One, that the dosage employed, was inadequate to prevent completely rats receiving a normal diet, was inadequate to balance the stimulation caused by a diet moderately deficient in sodium chloride. Under the conditions of their experiment the glomerulosa remained normal in width and lipid content.

SUMMARY AND CONCLUSIONS

To test the hypothesis that the electrolyte-regulating hormones of the adrenal gland are produced in the zona glomerulosa, the histochemical appearance of the rat's adrenal was studied in experiments

designed to disturb the normal ratio of sodium and potassium. Reduction of this ratio, accomplished by parenteral administration of potassium or dietary deficiency in sodium, caused cytological changes in the zona glomerulosa which indicated increased activity. After injection of potassium, the zone broadened and its lipid content decreased rapidly. With sodium deficiency, it became conspicuously wider and its cytoplasmic lipid droplets declined in size to eventual disappearance; these changes occurred in both normal and hypophysectomized rats. Similar but less marked changes occurred in the adrenal glands of rats deficient in both sodium and potassium.

Elevation of the sodium:potassium ratio, accomplished by administration of desoxycorticosterone acetate or dietary deficiency in potassium, caused cytological changes indicating inactivity of the glomerulosa. After these procedures, the glomerulosa cells became smaller, their lipid droplets enlarged in size but decreased in number, and the cytochemical reactions indicative of ketosteroids declined in intensity. With the hormone injection, all droplets ultimately disappeared.

These observations indicate that depression of the sodium:potassium ratio causes increased secretion by the cells of the zona glomerulosa and that elevation of this ratio causes decreased secretion or inactivity of the zone. These effects do not require the presence of the pituitary gland, but the mediating mechanism is unknown.

The administration of desoxycorticosterone (2 mg. daily) failed to prevent the glomerulosa changes accompanying sodium deficiency. Two possibilities to explain this anomalous result are: 1) the kidney may have been conserving sodium at nearly its maximal ability without the added desoxycorticosterone, or 2) the dose administered may have been too low.

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THE EFFECT OF INDUCED SECRETORY ACTIVITY ON THE CHOLESTEROL CONTENT OF THE IMMATURE RAT OVARY¹

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ALTHOUGH the metabolic degradation of steroid hormones has been extensively studied, the anabolic origin of these substances in the animal body has not received much attention. An important clue was furnished by Bloch (1945) who demonstrated that isotopically labelled cholesterol, fed to a pregnant woman, is in part converted to pregnandiol. The logical assumption is that such a conversion is by way of progesterone which, in the case of a woman in the late stages of pregnancy, may be assumed to have occurred in the placental tissues.

Another indication that cholesterol may be utilized for the synthesis of steroid hormones is afforded by a number of studies (Sayers, Sayers, Fry, White and Long, 1944; Sayers, Sayers, Liang and Long, 1946; Long, 1947; Levin, 1945) in which it was shown that stimulation of the adrenal cortex, at least in the initial stages, is accompanied by loss of stored cholesterol from this gland. Since such a loss of cholesterol is associated with various signs of adrenocortical hormonal secretion, the inference has been made that the stored cholesterol is utilized in the manufacture of the steroid hormones secreted by the cortex.

Both of the above lines of evidence as well as the molecular structural relationships suggest that ovarian secretion of estrogen and progesterone may also involve degradation of ovarian cholesterol. A study of ovarian cholesterol changes resulting from stimulation by gonadotrophic substances seemed likely to throw light on the subject. The present study, the results of which are presented below, was initiated for this purpose.

MATERIALS AND METHODS

Litters of female rats of the Long-Evans strain, 22 to 25 days old at the start of the experiment, were separated so as to distribute littermates, inso-

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far as was possible, among the various groups and also to keep the average body weights as uniform as possible. The animals were given a single subcutaneous injection of gonadotrophin and at various intervals thereafter, as indicated below, were sacrificed. The doses of the several gonadotrophic preparations were selected to evoke as nearly as possible equal stimulation as judged by ovarian weight response. Non-injected animals were always carried along as controls with the injected groups.

At the termination of each experiment, the individual animals were killed by ether anesthesia at the specific times indicated below. The ovaries and, in many cases, the uteri were immediately removed and carefully dissected free of fatty and connective tissues under the dissecting microscope. The presence of hyperemia, large follicles and corpora lutea were noted. Each pair of ovaries was weighed to the nearest one-tenth milligram and was immediately placed in a conical test tube to which was added 2 ml. of alcohol-acetone (1:1) mixture. The ovaries were finely minced in the alcohol-acetone by grinding with a pinch of washed sand and a closely fitting stirring rod. The extraction mixture was heated to boiling by immersing in a hot water bath and then allowed to cool to room temperature. The supernatant solution was transferred to a 5 or 10 ml. volumetric flask by means of a bulb pipette and the tissue residue was re-extracted several times more in the same manner with 1 ml. volumes of the same solvent, made up of the volumetric flask were allowed to come to room temperature, more to volume and after mixing, filtered through hot-alcohol-washed filter paper into a clean test tube. Aliquots of the clear solution were used for the determination of the total cholesterol content by Sperry's (1943) modification of the Schoenheimer-Sperry method. Because the cholesterol concentration of rat ovaries is small, it was necessary that the aliquot taken for analysis be as large as possible so as to increase the accuracy of the determination. For this reason, duplicate analyses on individual pairs of ovaries could not be made.

The following gonadotrophic substances were used: A commercial, partially purified lyophilized preparation of equine anterior pituitary gland sealed in sterile ampules. Each time the material was to be used, the contents of an ampule were dissolved in the correct amount of the accompanying solvent and the dose to be injected was calculated according to the label potency. As will be seen below, this preparation induced both follicular growth and luteinization.

A preparation of human chorionic (pregnancy urine) gonadotrophin, which, when assayed in this laboratory against the international chorionic gonadotrophin standard, had a potency of 660 i.u. per mg. In practice, this preparation was diluted with lactose so that small quantities could be conveniently weighed and solutions, in distilled water, were freshly prepared for each experiment. In most instances, the final solution contained 2 per cent lactose in addition to the gonadotrophin.

A preparation of post-menopausal urinary gonadotrophin made in this laboratory by a previously published method (Levin and Tyndale, 1937) per cent lactose assayed 1 mouse uterine unit (Levin and Tyndale, 1937) per 0.70 mg.

A commercial preparation of pregnant mare's serum gonadotrophin supplied as a dry powder in sterile ampules. This was freshly dissolved,

before use, in the appropriate amount of accompanying diluent according to the label potency.

RESULTS

The results obtained with a single injection of 10 rat units of hypophyseal gonadotrophin (APE) are presented in Table 1. It may be seen that there was a definite trend toward increase in ovarian

TABLE 1. EFFECT OF PITUITARY GONADOTROPHIN ON OVARIAN CHOLESTEROL OF IMMATURE RAT

Treatment	No.	Body weight	Ovarian weight		Ovarian cholesterol		
				Change	Cone.	Total	Change
		gm.	mg.	per cent	per cent	mg.	per cent
No treatment	60	44.0	12.7		1.160	0.147	
APE. 10 R.U. 1 hr.	8	44.4	13.9	+ 10	0.933*	0.130	- 12
3 hr.	7	44.4	14.0	+ 10	0.777*	0.109*	- 26
5 hr.	36	43.0	15.4*	+ 21	0.776*	0.118*	- 20
12 hr.	7	43.3	18.0*	+ 42	0.587*	0.105*	- 29
20 hr.	9	40.8	19.3*	+ 52	0.628*	0.121*	- 18
27 hr.	9	41.4	20.5*	+ 62	0.649*	0.133	- 10
48 hr.	6	45.0	34.3*	+170	0.800*	0.273*	+ 86
72 hr.	6	50.7	61.4*	+384	0.610*	0.373*	+154
96 hr.	5	52.0	48.5*	+282	0.749*	0.344*	+134

* Indicates value to differ significantly ($P = < 0.01$) from control value.

weight within an hour after the injection although this weight increase did not become statistically significant until 5 hours after the injection. The ovarian weight increase continued progressively until at least the 72nd hour. The ovarian cholesterol concentration was significantly decreased within one hour and continued to fall until 12 hours after the gonadotrophin injection. Thereafter, until the 96th hour, the cholesterol concentration remained approximately constant. That the early decrease in cholesterol concentration was not simply a consequence of the increased ovarian weight may be seen by inspection of the figures for the total cholesterol *content* of the ovaries. It is obvious that there was an actual loss of ovarian cholesterol within one hour after injection, becoming statistically significant by the third hour and remaining at a reduced level until at least 12 hours after the injection. Thereafter, cholesterol replenishment began, the ovaries contained almost twice as much cholesterol as did the controls and at 72 hours even more cholesterol had accumulated. At 96 hours, the total cholesterol content apparently was beginning to recede toward the control level. Thus, during the first 12 hours after a single injection of hypophyseal gonadotrophin, while the ovarian weight increased progressively, the ovarian cholesterol was as steadily lost. Thereafter, however, the continuing increase in ovarian weight was paralleled by an accumulation of cholesterol. This diphasic response is clearly demonstrated by a comparison of the percentage

change in ovarian weight and in total cholesterol content as is graphically represented in Figure 1.

Inspection of the ovaries and uteri of the animals treated with the hypophyseal gonadotrophin indicated that follicular stimulation, estrogen secretion and luteinization were occurring. From previous experience (Tyndale, Levin and Smith, 1938), it was known that with medium sized doses of post-menopausal urinary gonadotrophin excellent follicular stimulation and estrogen secretion, with little or no luteinization, may be achieved. In order, therefore, to determine whether the cholesterol loss is associated with follicular stimulation and estrogen secretion as opposed to luteinization, an experiment, similar to that described above, was carried out using post-menopausal urinary gonadotrophin (CU). The results of this experiment are presented in Table 2.

TABLE 2. EFFECT OF POSTMENOPAUSAL GONADOTROPHIN ON OVARIAN CHOLESTEROL OF IMMATURE RAT.

Treatment	No.	Body weight	Ovarian weight				Ovarian cholesterol		
			Change		Conc.		Total	Change	
		gm.	mg.	per cent	per cent	mg.		per cent	
No treatment		44.0	12.7			1.160	0.147		
C.U. 10 mg. L531	60	50.7	13.3			1.020	0.136		
1 hr.	6	50.4	15.4*	+ 5		0.958*	0.146	- 7	
3 hr.	7	44.3	13.9	+ 21		1.082	0.148	- 1	
5 hr.	18	46.3	17.3*	+ 10		0.968*	0.167	+ 1	
13 hr.	7	40.3	21.4*	+ 36		0.812*	0.174	+14	
24 hr.	4	39.8	27.8*	+ 68		0.663*	0.185*	+18	
48 hr.	5	38.5	20.4*	+119		0.704*	0.143	+26	
72 hr.	4			+ 60				- 3	

* Indicates value to differ significantly ($P = < 0.01$) from control value.

The data show a definite and progressive increase in ovarian weight similar to that observed after injection of the pituitary gonadotrophin. The increase in ovarian weight after CU administration was not, however, sustained for as long as after APE, the maximum increase with the former being attained at 48 hours after a single injection as compared to the maximum at 72 hours with the APE.

The ovarian cholesterol concentration showed no consistent change after post-menopausal urine injection until the 13th hour when a significant decrease was found to have occurred. This diminution in concentration became progressively greater at the 24th and 48th hours. At the 72nd hour, the cholesterol concentration began to return toward the original control level.

It is to be noted that after the injection of post-menopausal gonadotrophin the changes in cholesterol concentration may be directly correlated with the changes in ovarian weight. The inverse proportionality between ovarian weight and ovarian cholesterol concentration is quite striking and indicates that little change in absolute amount of cholesterol is actually occurring. This impression is confirmed by inspection of the figures for the total content of cholesterol

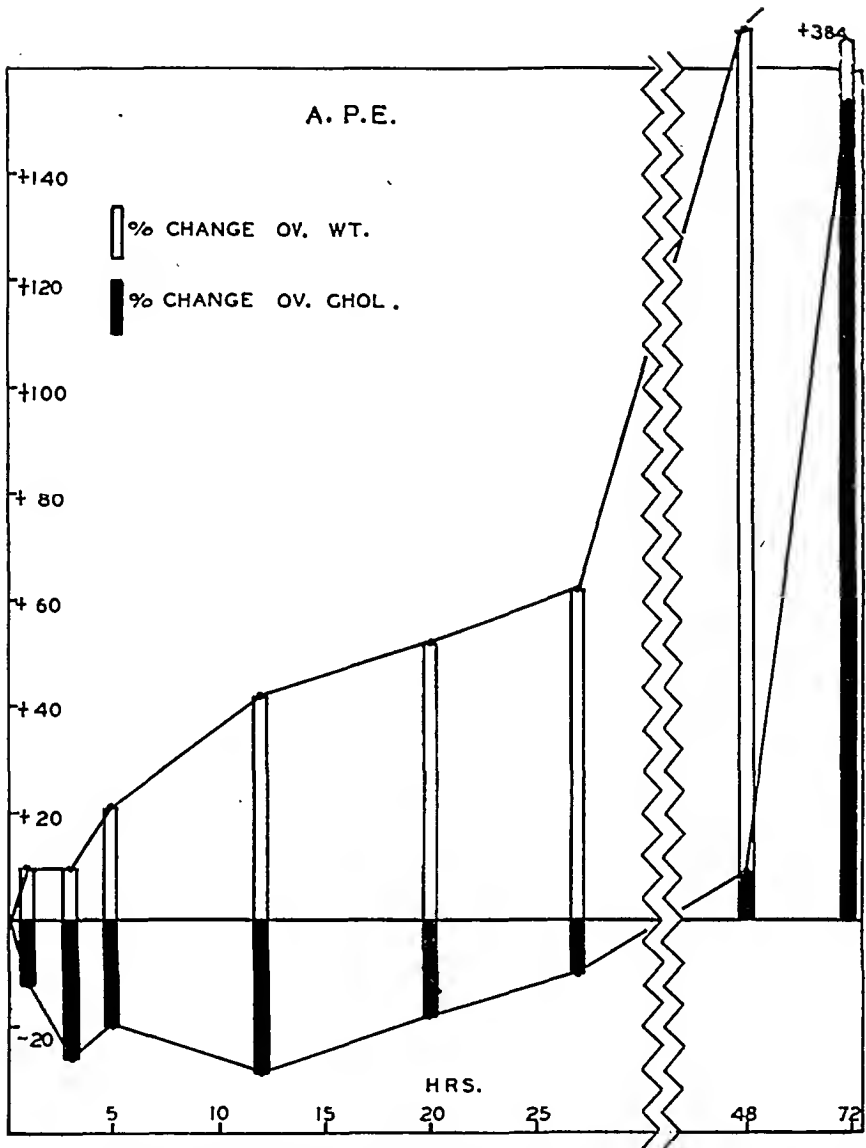


FIG. 1. Effect of Pituitary Gonadotrophin on the Weight and Cholesterol Content of the Immature Rat Ovary. A single subcutaneous injection of 10 R.U. was made at 0 hours.

per pair of ovaries. It is evident that at no time except at one hour after injection was there a decrease in the absolute amount of cholesterol and even that decrease was not statistically significant. The early effect of this gonadotrophin therefore differs from that of APE in that the CU does not cause a loss of ovarian cholesterol. The later effects, 24 to 48 hours after injection, are similar to those of APE—i.e., both types of gonadotrophin cause an accumulation of cholesterol at these times. The graphic representation of this data (Figure 2) clearly shows the early cholesterol response to differ from that obtained with APE.

The fact that APE caused a loss of ovarian cholesterol whereas

the post-menopausal urinary gonadotrophin did not do so presented several alternative possibilities. One possibility was that the APE might contain adrenotropic hormone which has previously been shown (Sayers, Sayers, Fry, White and Long, 1944) to cause a loss of adrenal cholesterol and conceivably might also cause a loss of ovarian cholesterol. To determine the validity of such a possibility, removed 9 hours after injection and the cholesterol level determined. There was no change as compared to untreated rats, indicating that this preparation contained little or no adrenotropic hormone. In addition, several other groups of rats were treated with a preparation of adrenotropic hormone (ACTH) and a further group was exposed

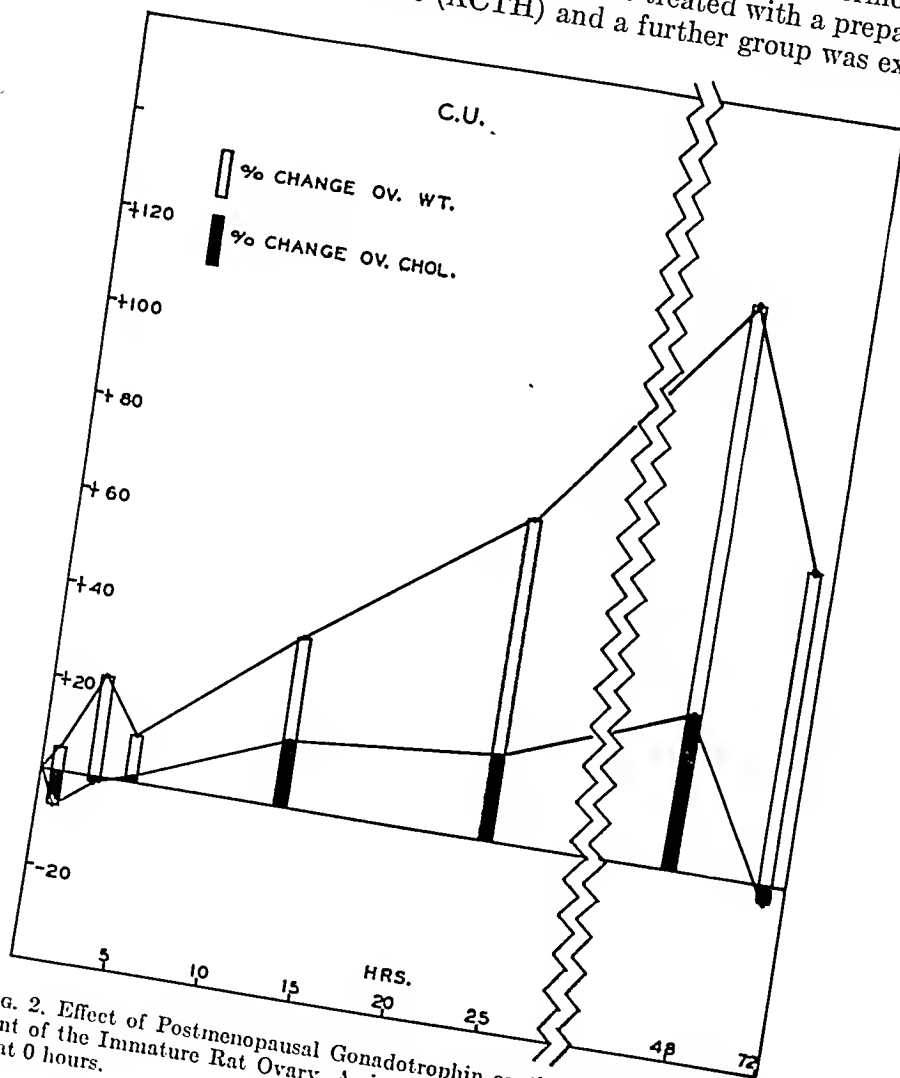


FIG. 2. Effect of Postmenopausal Gonadotrophin on the Weight and Cholesterol Content of the Immature Rat Ovary. A single subcutaneous injection of 10 mg. was made at 0 hours.

to low temperature for 5 hours, a procedure reported (Fry, cited by Sayers *et al.*, 1944; Long, 1947; Levin, 1945) to cause loss of adrenal cholesterol by causing a discharge of adrenotrophic hormone by the animal's own pituitary gland. The ovaries and adrenals were removed at appropriate times and the cholesterol contents determined. The data are summarized in Table 3. It is evident that neither injection of ACTH nor exposure to cold caused any significant change in the ovarian cholesterol level although the ACTH, when administered in adequate dose, did cause a marked loss of adrenal cholesterol.

TABLE 3. EFFECT OF ADRENOTROPHIN ON OVARIAN AND ADRENAL CHOLESTEROL OF IMMATURE RAT

Treatment	No.	Body weight gm.	Ovarian weight		Ovarian cholesterol			Adrenal	
			mg.	Change per cent	Conc. per cent	Total mg.	Change per cent	Weight mg.	Cholest. per cent
No treatment	60	44.0	12.7		1.160	0.147		13.3	3.27
ACTH 0.5 mg. 5 hr.	5	43.0	13.9	+9	1.143	0.158	+7	13.8	3.43
5.0 mg. 5 hr.	5	46.0	13.0	+2	1.191	0.155	+5	13.7	1.62
Cold 5°C.	7	45.9	13.1	+3	1.162	0.152	+3		

These findings eliminate the possibility that the loss of ovarian cholesterol resulting from administration of the APE may be ascribed to any adrenotrophic hormone which it might contain. Consequently, the difference in response to APE and post-menopausal gonadotrophin cannot be due to the presence or absence of adrenotrophin.

In an attempt to further elucidate the ovarian cholesterol changes, the effect of human chorionic gonadotrophin (PU) was studied. This substance is known to have physiological properties similar to those ascribed to the pituitary luteinizing hormone and, in hypophysectomized animals, is devoid of any follicle stimulating properties. The results obtained with this substance, summarized in Table 4, are very similar to those obtained with the unfractionated pituitary extract. The same early loss of ovarian cholesterol, coincident with increasing ovarian weight was found and this cholesterol loss was

TABLE 4. EFFECT OF CHORIONIC GONADOTROPHIN ON OVARIAN CHOLESTEROL OF IMMATURE RAT

Treatment	No.	Body weight gm.	Ovarian weight		Ovarian cholesterol		
			mg.	Change per cent	Conc. per cent	Total mg.	Change per cent
No. Treatment	60	44.0	12.7		1.160	0.147	
P.U. 300 γ L759 3 hr.	9	41.7	15.0*	+ 18	0.779*	0.119*	-19
5 hr.	14	44.3	14.5*	+ 14	0.764*	0.110*	-25
12 hr.	11	40.7	15.3*	+ 20	0.680*	0.103*	-30
24 hr.	8	39.0	16.4*	+ 29	0.776*	0.126?	-14
47 hr.	7	46.9	25.6*	+102	0.402*	0.103*	-30
71 hr.	7	45.1	28.4*	+124	0.574*	0.160	+ 9
97 hr.	5	47.0	22.2*	+ 75	1.154	0.256*	+74

* Indicates value to differ significantly ($P = < 0.01$) from control value.

associated with a pronounced decrease in the concentration of ovarian cholesterol. It is to be noted that the duration of the cholesterol loss is at least 48 hours, a considerably longer period than observed after APE injection.

During the later periods, e.g., 71 hours or more after the gonadotrophin administration, there is an accumulation of cholesterol in the ovaries. Perhaps because of the waning of the stimulatory effects of the single injection of gonadotrophin, at 96 hours the cholesterol concentration had returned to the original pre-injection level, even though the total cholesterol content was far above the control value. The graphic representation of these results, shown in Figure 3, indicates that the entire cycle of ovarian cholesterol changes is quite similar to that seen after APE injection (Figure 2).

TABLE 5. EFFECT OF PREGNANT MARE'S SERUM GONADOTROPHIN ON OVARIAN CHOLESTEROL OF IMMATURE RAT

Treatment	No.	Body weight gm.	Ovarian weight				Ovarian cholesterol		
			mg.	Change per cent			Conc. per cent	Total mg.	Change per cent
No treatment	60	44.0	12.7				1.160	0.147	- 3
Antex 500 i.u. 5 hr.	13	47.8	13.8	+ 9			1.074	0.143	- 20
24 hr.	12	39.4	15.2*	+ 20			0.813*	0.118*	- 5
49 hr.	6	44.6	46.6*	+267			0.326*	0.154	+ 5
72 hr.	7	48.1	65.3*	+414			0.450*	0.295*	+100

* Indicates values to differ significantly ($P = < 0.01$) from control value.

Interesting results were also obtained with the gonadotrophin of pregnant mare's serum (PMS) (Table 5). Twenty-four hours after a single injection of 500 i.u. the ovaries were moderately enlarged (20 per cent increase). During the same interval, 20 per cent of the ovarian cholesterol was lost. As a result of these coincident changes in opposite directions, the reduction in cholesterol concentration is accentuated.

The later phases of the reaction are characterized by further marked gain in ovarian weight and a slow replenishment of the cholesterol content. Because of the relatively great weight increase, the cholesterol remains at a very low concentration despite the fact that the total amount of cholesterol is considerably elevated, being double the control level at 72 hours after the injection. These responses therefore are similar to those noted with chorionic gonadotrophin and pituitary extract. However, the very early response with PMS differs from those noted above in that at 5 hours after the injection there is little change in ovarian cholesterol whereas with the other luteinizing gonadotrophins a definite cholesterol loss was observed at 1 hour and certainly at 3 and 5 hours after the injection.

DISCUSSION

A complete interpretation of the above results is not possible at the present time. It is evident that with certain gonadotrophins, such

as those of unfractionated pituitary extract, of human pregnancy urine and of pregnant mare's serum, the rat ovary responds by a rapid and pronounced loss of cholesterol in spite of a distinct increase in ovarian weight. During the later stages of the gonadotrophic

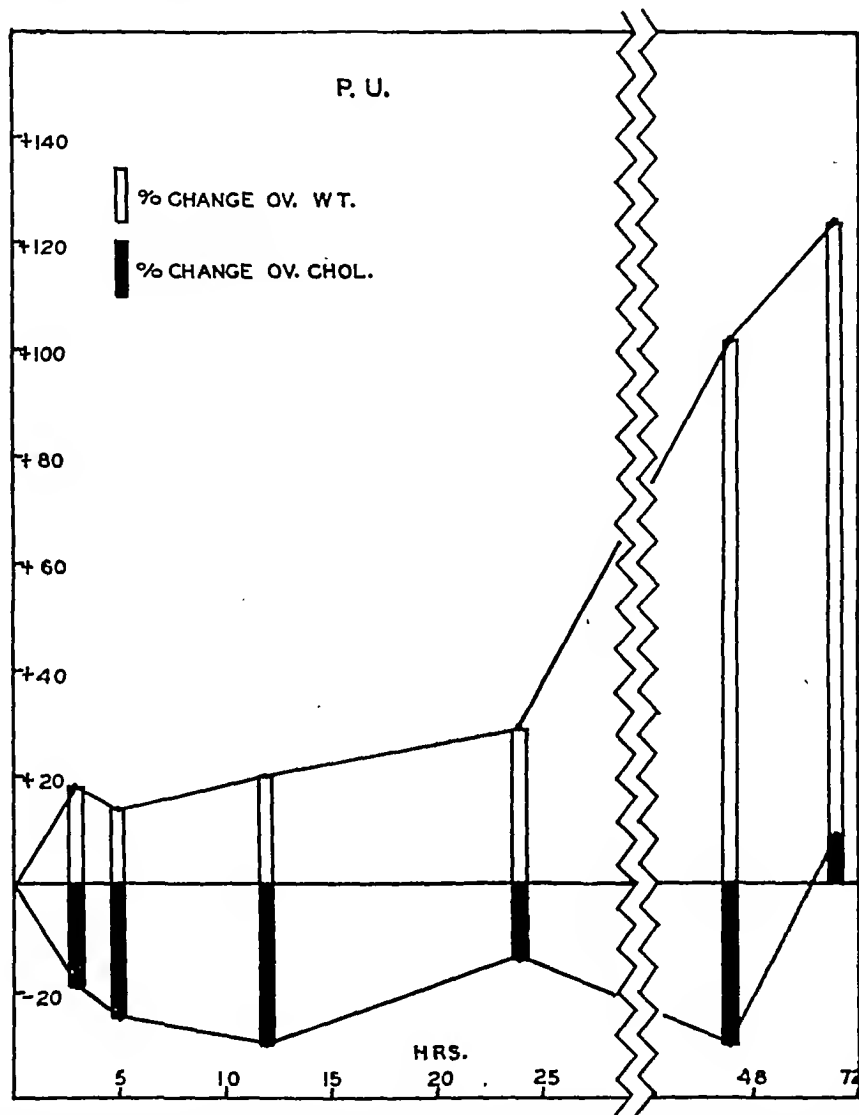


FIG. 3. Effect of Human Chorionic Gonadotrophin on the Weight and Cholesterol Content of the Immature Rat Ovary. A single subcutaneous injection of 300 μ g. (200 i.u.) was made at 0 hours.

stimulatory action, the ovarian cholesterol is apparently replenished and the total quantity of cholesterol finally greatly exceeds the original level although, because of continuing increase in ovarian weight, the concentration remains considerably below the pre-injection level until at least the 96th hour.

On the other hand, with suitable doses of gonadotrophin from human post-menopausal urine, the initial phase of cholesterol loss

does not occur. During the later stages of the gonadotrophic stimulatory action there is a considerable increase in the amount of ovarian cholesterol. That this increase is not proportional to the increase in ovarian weight, however, is indicated by the fact that although the absolute amount of cholesterol increases, the concentration falls due to the coincidental rise in ovarian weight. It would appear that although cholesterol deposition occurs it does not keep pace with the deposition of other components of the newly formed tissue.

The marked differences between the early response to APE, PU and PMS as opposed to CU cannot be clearly interpreted. It has been well established that all these gonadotrophins cause estrogen secretion. Even in hypophysectomized rats, in which it produces no sign of follicular stimulation, PU causes massive estrogen secretion (Leonard and Smith, 1934). Likewise APE, PMS and CU are well known to be effective stimulators of estrogen secretion. It is clear that the differences in ovarian cholesterol response cannot be ascribed to differences in ability to cause estrogen secretion.

A more tenable explanation is that based on the known differences in the luteinizing potentialities of the two types of gonadotrophin. It is well established that chorionic gonadotrophin is predominantly luteinizing in its action (Leonard and Smith, 1934). Likewise, it has long been known that unfractionated pituitary extract has strong luteinizing effects (Smith, 1935). Although equine gonadotrophin in small or medium doses does not cause massive luteinization, it does produce thecal lutein changes even when minimal doses are administered (Leatham, 1939). These substances are shown in the present report to cause a rapid and marked loss of ovarian cholesterol.

Post-menopausal gonadotrophin, on the other hand, has little if any luteinizing action unless relatively high doses are administered (Tyndale, Levin and Smith, 1938). This gonadotrophin, the only one not having a luteinizing action in the doses administered, did not cause a loss of ovarian cholesterol in the present experiments. It is therefore tempting to associate the early loss of ovarian cholesterol with the luteinizing gonadotrophic action and consequently with the secretion of progesterone.

There are, however, several obstacles to the easy acceptance of such a hypothesis. One disturbing factor is that of time relationships. It has always been the impression of the authors that with gonadotrophins having both follicle stimulating and luteinizing activities, the follicle stimulation and estrogen secretion are seen relatively early whereas luteinization is a later phenomenon. In the present experiments, however, it is shown that the cholesterol loss occurs very soon (3-24 hours) after the gonadotrophin administration. If the cholesterol loss is related to the secretion of progesterone then the luteinizing action must be very prompt indeed. That this may actually be the case is indicated by the preliminary report of Bradbury

(1948) who studied the early ovarian histological response to various gonadotrophins and found that lutein changes, when they occur, are seen shortly after administration of all gonadotrophins he tested with the single exception of CU.

A more serious obstacle to a simple interpretation of the present results is that of the concept of luteotrophic action of the lactogenic hormone. It has been reported (Evans, Simpson and Lyons, 1941; Evans, Simpson, Lyons and Turpeinin, 1941) that lutein tissue does not secrete progesterone unless activated by a luteotrophic hormone, thought to be identical with the lactogenic hormone. Recently, Everett (1947) has claimed that by use of histological techniques he could demonstrate a marked loss of cholesterol from corpora lutea as a consequence of administration of lactogenic hormone to rats.

The possibility existed that in the present experiments the loss of ovarian cholesterol was the result of the action of luteotrophin, either contained in the administered gonadotrophin or secreted by the animal's own pituitary gland in response to the sudden stimulatory process initiated by the administered material. In order to test the Everett hypothesis a preliminary set of experiments was set up. In a small series of rats the ovarian cholesterol level was caused to increase by injecting pituitary gonadotrophin as in the experiments of Table 1. Seventy-two hours after the injection of pituitary gonadotrophin, 200 i.u. of lactogenic hormone was injected into half of the animals, the remaining half being retained as controls. Twenty-four hours after the injection of lactogenic hormone (i.e., 96 hours after the APE injection) the animals were sacrificed. Although the animals injected with lactogenic hormone showed a slightly lower ovarian cholesterol level, the difference between these and the control APE injected animals was certainly not significant. Furthermore, the ovarian cholesterol content of animals injected only with 200 i.u. of lactogenic hormone and sacrificed 24 hours later was identical with that of uninjected control animals. These results therefore provide no evidence to indicate that the loss of ovarian cholesterol is due to the luteotrophic action of the gonadotrophin.

The present findings are in good agreement with those of Claesson and Hildarp (1947a; 1947b) who, by histochemical means, have found that coitus as well as administration of chorionic or pregnant mare's serum gonadotrophin causes a loss of "cholesterol" from the ovaries of rats and rabbits. However, our interpretations do not agree with those of Claesson and Hildarp. They suggest that the "cholesterol" is lost from the ovary during the process of estrogen secretion and, in fact, refer to it as the "oestrogen precursor." This conclusion is difficult to understand especially since they show that ovaries of rabbits in the estrus state (due to continuous secretion of estrogen?) contain large amounts of "cholesterol" and this "cholesterol" is rapidly removed by procedures (coitus, injection of PU or PMS) which cause ovulation and lutein change.

The changes in ovarian cholesterol described above are reminiscent of those obtained (Sayers *et al.* 1944; Sayers *et al.* 1946; Long, 1947a) in adrenal cholesterol following administration of adrenotropic hormone. In the case of the adrenal, a good correlation could be made between loss of cholesterol and various indications of secretion of cortical hormone thereby providing reasonable grounds for the hypothesis that the adrenal cholesterol is used for the synthesis of adrenocortical hormone. In the present experiments a good correlation between ovarian secretion and loss of ovarian cholesterol can be made. The situation is complicated, however, by the fact that the ovary consists of two distinct secretory organs which produce two different types of hormone. On the basis of presently available evidence it appears most likely that it is during the phase of luteal secretion that the cholesterol depletion occurs. To definitely establish that this is actually the case will require further investigation, particularly in hypophysectomized animals in which the unknown contribution of the animal's own hypophysis is definitely eliminated.

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SUMMARY

A single injection of equine pituitary extract, of human chorionic gonadotrophin or of gonadotrophin from pregnant mare's serum, administered to immature female rats, results in a marked loss of cholesterol from the ovaries. This loss is first evident 3 hours after the gonadotrophin injection and the minimum cholesterol level is attained at approximately 12 hours. Thereafter, cholesterol accumulation occurs until, at 48 to 72 hours after the injection, the total amount present in the ovaries greatly exceeds the original control level. However, because of the coincident and disproportionate increase in ovarian weight, the cholesterol concentration remains far below that of control ovaries.

In contrast to the above results, a single injection of post-menopausal urinary gonadotrophin, although producing a similar increase in ovarian weight, does not cause a loss of ovarian cholesterol. During the later stages of its action, it does cause an increase in total cholesterol.

sterol content but, as above, the cholesterol concentration remains at a low level.

Experiments designed to test the participation of adrenotrophic and luteotrophic hormones in causing loss of ovarian cholesterol indicate that neither of these hormones is involved. Rather, it seems likely, though not proven, that the loss of cholesterol is associated with luteinizing action and therefore with secretion of progesterone by the stimulated ovaries.

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THE RELATIONSHIP BETWEEN THYROID ACTIVITY AND THE LEVEL OF PSEU- DO-CHOLINESTERASE IN THE PLASMA OF RATS¹

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IT HAS BEEN shown in this laboratory (Mundell, 1944) that the difference in the ability of male and female rat plasma to hydrolyze acetylcholine is due to the higher level of pseudo-cholinesterase in the plasma of the latter. Sawyer and Everett (1946) have presented evidence that the level of pseudo-cholinesterase in the plasma of rats parallels the estrogen level of these animals and in a later paper (Everett and Sawyer, 1946a) these authors concluded that the effect of estrogenic substances on the level of pseudo-cholinesterase in the rat is mediated through the hypophysis.

To extend the examination of the relationship of hormones to the level of pseudo-cholinesterase in rat plasma, an investigation into the effect of thyroid function on the activity of this enzyme was undertaken. A preliminary report of some of the observations recorded in this series of experiments has already appeared (Hawkins, Mendel and Nishikawara, 1948).

Antopol, Tuelman and Schiffrin (1938) have reported that the acetylcholine-hydrolyzing ability of human serum is increased beyond the normal range in hyperthyroid patients and that upon treatment the average activity of their sera approaches that obtained in normal subjects. Similar results were obtained by Faber (1943). Since the activity of human serum towards acetylcholine is due mainly to the activity of pseudo-cholinesterase (Mendel, Mundell and Rudney, 1943), the elevation observed by Antopol and his co-workers in cases of human hyperthyroidism presumably is due to the increased activity of this enzyme. In contrast to the results obtained in patients, Talesnik and Jimenez observed a depression in the cholinesterase activity of the sera of hyperthyroid rats, whereas in cats and rabbits this decrease was less evident (Hoffman and Hoffmann, 1944). However, no details of the experimental procedure, the substrate employed or its concentration were outlined.

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MATERIALS AND METHODS

Adult male Wistar rats were used as experimental animals. They were maintained on a diet of chow. Following the experimental period, oxalated plasma was obtained by exsanguination through the jugular vein. The activity of the cholinesterases (ChE) was estimated manometrically at 37.5°C. in a medium containing 0.025 M NaHCO_3 saturated with 5% CO_2 in N_2 (pH 7.4). The activity of true cholinesterase was determined with acetyl- β -methylcholine (Meh)² in a concentration of 0.03 M and that of pseudo-cholinesterase with benzoylcholine (Beh) in a concentration of 0.006 M, according to the method of Mendel, Mundell and Rudney (1943). One cc. of plasma was used in the estimation of enzymatic activity in all cases and the values appearing in the subsequent tables represent the number of cubic millimetres of CO_2 evolved over a 20-minute period.

EXPERIMENTAL

A. *The Effect of Thyroidectomy on the Activity of the Cholinesterases in Male Rat Plasma.*

Thyroidectomy was carried out on 16 animals, as outlined by Griffith and Farris (1942). Removal of varying amounts of parathyroid tissue was unavoidable. Therefore, in order to offset the possible effects of parathyroidectomy 2 ml. of a 5% calcium gluconate solution were administered daily for one week. Fourteen days after operation the animals were sacrificed; this time interval was sufficient for the recovery of the level of pseudo-cholinesterase from the decline resulting from operation (Everett and Sawyer, 1946b). A control group of 27 normal animals was used for comparison. The activity of the cholinesterases in the plasma of these animals is outlined in Table 2. When the values appearing in this table are compared with those obtained in normal animals (Table 1) it will be seen that while the activity of the true cholinesterase of the treated animals remains unchanged, that of the pseudo-cholinesterase shows a significant rise. Thyroid removal, therefore, appears to cause an increase in the level of pseudo-cholinesterase in the plasma.

B. *The Effect of Thiouracil Treatment on the Level of Pseudo-Cholinesterase in the Plasma of Male Rats.*

Since thyroidectomy is accompanied by the unavoidable removal of parathyroid tissue, it was necessary to exclude the possibility that the rise in pseudo-cholinesterase in the plasma following thyroidectomy was due to parathyroidectomy rather than thyroidectomy. Accordingly, a group of 15 rats was treated with thiouracil. These animals received a water ration containing 0.06% thiouracil for a period of 23-27 days. The level of pseudo-cholinesterase in their plasma following this treatment is given in Table 3. The results indicate that the rise in pseudo-cholinesterase obtained after thyroidectomy (Table

² Merck's Mecholyl.

TABLE 1. ACTIVITY OF THE CHOLINESTERASE IN NORMAL MALE RAT PLASMA

True ChE. Substrate: Mch	Pseudo-ChE. Substrate: Bch
65.7	29.8
58.2	35.4
38.2	37.4
63.5	54.9
47.7	23.2
82.9	51.9
67.6	31.0
64.9	32.8
53.1	30.0
69.9	58.9
78.4	51.4
68.0	46.2
56.4	42.6
56.9	40.1
38.1	23.1
64.0	43.9
67.5	37.7
64.9	35.4
41.0	48.6
74.3	75.4
63.1	29.3
73.0	41.5
79.1	38.7
66.2	30.0
77.0	55.5
73.4	42.3
60.5	40.9
51.0	—
Aver. 63.0	41.0
S.E. 2.1	2.3

TABLE 2. THE EFFECT OF THYROIDECTOMY ON THE LEVELS OF THE CHOLINESTERASE IN MALE RAT PLASMA

True ChE. Substrate: Mch	Pseudo-ChE. Substrate: Bch
56.2	87.7
77.4	158.7
71.2	79.2
50.9	55.4
70.6	140.2
58.3	137.9
60.9	156.1
50.8	67.9
48.4	63.6
57.6	96.4
69.0	81.5
65.3	114.0
60.8	98.3
60.3	64.8
—	56.7
—	94.5
Aver. 61.3	97.1
S.E. 2.3	8.7
t 0.31	7.7
P 0.76	0.38×10^{-12}

2) is due to thyroid rather than to parathyroid removal. Statistically, the rise in pseudo-cholinesterase activity resulting from both thyroidectomy and treatment with thiouracil is highly significant.

TABLE 3. EFFECT OF THIOURACIL TREATMENT ON THE LEVEL OF PSEUDO-CHOLINESTERASE IN MALE RAT PLASMA

Pseudo-ChE. Substrate: Bch	
159.6	
228.1	
142.5	
84.6	
153.8	
70.0	
52.8	
102.4	
51.2	
77.8	
84.0	
130.4	
182.8	
114.3	
125.9	
Aver.	117.3
S.E.	10.6
t	9.1
P	1.2×10^{-16}

C. *The Effect of Thyroxine Administration on the Level of the Cholinesterases in Male Rat Plasma*

In vitro experiments revealed that thyroxine has no effect on the activity of pseudo-cholinesterase. However, to determine whether an increase in circulating thyroxine would exert an effect on the level of plasma pseudo-cholinesterase the reverse of that observed after thyroidectomy, one milligram of thyroxine³ per 100 gm. body weight was administered to 15 male rats every other day for a period of 14-16 days. The animals were then sacrificed in the usual manner. The activities of their plasma cholinesterases appear in Table 4. It will be seen that the administration of thyroxine to normal male rats causes a depression in the level of plasma pseudo-cholinesterase which is highly significant. On the other hand, no appreciable deviation from the normal occurs in the level of plasma true cholinesterase. Thyroxine administration, therefore, exerts an effect on plasma pseudo-cholinesterase activity which is the direct opposite of that resulting from thyroid deficiency.

D. *The Effect of Treatment with a Combination of Thiouracil and Thyroxine on the Activity of Plasma Pseudo-Cholinesterase in Male Rats*

An experiment was undertaken to ascertain whether thyroxine administration would prevent the rise in plasma pseudo-cholinesterase

³ Roche-Organon (Synthetic Thyroxine).

which is observed when normal male rats are treated with thiouracil. Fifteen male rats were given a water ration containing 0.06% thiouracil for a period of 23-25 days. In addition, 20% of thyroxine per 100 gm. body weight was administered daily. This dose was felt to be adequate to supply daily requirements, in view of the findings of Meyer and Wertz (1939) that thyroidectomized rats are almost thirty times

TABLE 4. EFFECT OF THYROXINE ADMINISTRATION ON THE ACTIVITY OF THE CHOLINESTERASE OF MALE RAT PLASMA

True ChE. Substrate: Mch	Pseudo-ChE. Substrate: Bch
67.3	23.9
58.5	22.8
70.7	24.6
46.3	30.0
65.3	29.2
55.5	28.4
73.2	21.9
78.0	24.5
68.0	18.9
56.7	20.0
56.0	29.7
52.9	36.9
54.3	32.4
61.2	47.6
49.6	27.6
Aver. 60.9	27.9
S.E. 2.3	1.9
t 0.59	3.85
P 0.56	2.2×10^{-4}

more sensitive to thyroxine than normal animals. The effect of the above experimental procedure on the level of pseudo-cholinesterase in the plasma of these animals is presented in Table 5. The fact that no significant deviation from the normal occurs indicates that the administration of thyroxine prevents the rise in plasma pseudo-cholinesterase levels induced by treatment with thiouracil.

DISCUSSION

Our observation that an elevated thyroxine level induces in rats a depression in the plasma pseudo-cholinesterase activity is at variance with the findings of Antopol, Tuchman and Schiffrin, who reported an elevation in the cholinesterase level of hyperthyroid patients. This difference in species reaction is unexplained and further experiments will be required for its elucidation.

Whether the effects observed in rats are due directly to the thyroid hormone or whether they are mediated through the pituitary has not been fully determined. Although Sawyer and Everett found the potentiating effect of estrogenic substances on rat plasma pseudo-cholinesterase to be mediated through the hypophysis, preliminary experiments on the thyroid-hypophyseal interrelationship seem to indi-

cate that the effect of thyroxine on the activity of the enzyme is not exerted through hypophyseal intermediation. There is, however, a possibility that the liver is involved in some way in the action of thyroxine on pseudo-cholinesterase since Sawyer and Everett (1947) have shown that this organ is the main site of pseudo-cholinesterase formation. These two points still remain to be settled.

TABLE 5. THE EFFECT OF COMBINED TREATMENT WITH THYROXINE AND THIOURACIL ON THE LEVEL OF PSEUDO-CHOLINESTERASE IN MALE RAT PLASMA

Pseudo-ChE. Substrate: Bch	
28.8	
30.9	
28.8	
26.3	
32.0	
41.1	
38.0	
45.8	
37.5	
57.4	
50.0	
32.5	
28.8	
63.3	
38.2	
Aver.	38.6
S.E.	2.9
t	0.65
P	0.54

Although thyroxine seems to exert an inhibitory action on the level of plasma pseudo-cholinesterase, it is difficult to venture a suggestion as to what this implies. The function of pseudo-cholinesterase is still obscure. Sawyer and Everett (1947) have advanced the hypothesis that since the enzyme hydrolyzes not only acetylcholine but such simple esters as tributyrin and methyl butyrate, its principal function might be that of a simple lipase concerned with lipid metabolism. It has been suggested (Hoffmann and Hoffmann, 1944; Handler 1948) that the thyroid plays a major role in phospholipid metabolism and it is a well-known fact that changes in the level of thyroid activity are accompanied by changes in phospholipid and lipid metabolism. Thus, one common association between thyroid activity and plasma pseudo-cholinesterase activity may be found in a mutual relationship with lipid and phospholipid metabolism but this association may be merely fortuitous.

SUMMARY

A depression in the level of thyroxine in normal male rats induced by thyroidectomy or by treatment with thiouracil results in an elevation in the activity of pseudo-cholinesterase in the plasma of these

animals. Statistically, the rise in the activity of this enzyme is highly significant.

An elevation in the level of thyroxine, on the other hand, leads to a depression in the activity of plasma pseudo-cholinesterase which is also highly significant. Administration of thyroxine prevents the rise in pseudocholinesterase which is induced by thiouracil treatment.

The level of plasma true cholinesterase is not affected by the above procedures.

The significance of these findings is briefly discussed.

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THE INHIBITORY ACTION OF EXCESSIVE IODIDE UPON THE SYNTHESIS OF DIIODOTYROSINE AND OF THYROXINE IN THE THYROID GLAND OF THE NORMAL RAT¹

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BOTH CHEMICAL (Harington, 1944, 1945; von Mutzenbecher, 1939; Block, 1940; Reineke and Turner, 1942) and biochemical evidence (Perlman *et al.*, 1941; Morton *et al.*, 1943; Taurog *et al.*, 1947; Leblond, 1942) has shown that at least 2 steps are involved in the synthesis of thyroxine: 1) iodination of tyrosine to form diiodotyrosine and 2) coupling of 2 diiodotyrosine molecules to form thyroxine. While the exact nature of step 1 is not well understood, it is apparent that the iodide reaching the thyroid gland from the blood must be oxidized either to I_2 or to HIO before it can be incorporated into the phenolic group of tyrosine. The subsequent coupling of 2 diiodotyrosine molecules is believed by both Harington (1945) and Johnson and Tewkesbury (1942) to be an oxidative reaction in which the oxidized form of iodide (I_2 or HIO) plays a significant role.

In a recent communication (Wolff and Chaikoff, 1948a, 1948b) it was demonstrated that the administration of relatively large doses of inorganic iodide to *normal* rats blocked the formation of organic iodine in the thyroid gland. This block was related to the level of plasma iodine. So long as the level of plasma iodine remained above 20–35 gamma per cent, no appreciable conversion of inorganic iodide to organic forms occurred in the gland (Wolff and Chaikoff, 1948c). In keeping with the considerations presented above, excessive iodide could thus exert its inhibitory effect by blocking 1) the iodination of tyrosine and/or 2) the coupling mechanism involved in the formation of thyroxine from diiodotyrosine. These possibilities are examined in the present communication.

EXPERIMENTAL

Male rats of the Long-Evans strain, weighing 170–240 gms., were used throughout. Their mean body and thyroid weights are recorded

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in Table 1. They were fed a diet containing 0.3% iodine per gm. This diet consisted of 68.5 per cent wheat, 5 per cent casein, 10 per cent fish meal, 10 per cent alfalfa, 1.5 per cent sodium chloride, and 5 per cent sardilene (a fish oil that supplied vitamins A and D). Amounts of iodide as KI labeled with I^{131} varying from 5 to 100% were injected intraperitoneally (Tables 2 and 3). The rats of each experiment were sacrificed at single interval. In experiments 2, 5, and 6, this interval was 4 hours; in experiment 1, it was 4.6 hours.

TABLE 1. BODY WEIGHTS AND THYROID WEIGHTS OF RATS

Experiment	Number of rats	Body weights	Thyroid weights
1	9	gm.	mg.
2	10	$214 \pm 19^*$	$21 \pm 0.3^*$
5	10	188 ± 9	17 ± 0.2
6	13	174 ± 8	17 ± 0.2
		177 ± 7	22 ± 0.3

* Standard deviation.

The animals were anesthetized with sodium pentobarbital (20. mg. per rat) and then exsanguinated. The thyroid glands were rapidly excised, weighed, and immediately thereafter transferred to 1 cc. of cold 10 per cent trichloroacetic acid (T.C.A.). The inorganic (T.C.A.-soluble) fraction was separated as previously described (Wolff and Chaikoff, 1948b), and discarded. The organic fraction (T.C.A.-insoluble) was separated into diiodotyrosine-like and thyroxine-like fractions by the method described elsewhere (Taurog and Chaikoff, 1946a).

RESULTS AND DISCUSSION

In each experiment (Table 2) the amounts of I^{127} injected were chosen so that for the duration of the experiment (i.e., 4 or 4.6 hours) the concentration of plasma iodine remained well above or well below the critical level previously shown to produce inhibition of organic binding of iodine in the gland (Wolff and Chaikoff, 1948b). The non-inhibitory doses were either 5% (experiments 1 and 2) or 10% of I^{127} (experiments 5 and 6), whereas 50 or 100% were injected to obtain inhibition. The diiodotyrosine and thyroxine (I^{127}) contents of the thyroid gland, as well as the percentages of the injected I^{131} recovered in these 2 fractions, are recorded in Table 2.

1. Calculation of Newly Synthesized Diiodotyrosine

Since both plasma and thyroid glands of the rats fed our low-iodine diet contained negligible amounts of inorganic iodide before the injections were made (Taurog and Chaikoff, 1946b, 1947), and furthermore since the amounts of I^- injected were large with respect

TABLE 2. DIODOTYROSINE-LIKE, AND THYROXINE-LIKE FRACTIONS FOUND IN THYROIDS OF RATS INJECTED WITH VARIOUS AMOUNTS OF LABELED IODIDE

Expt.	Iodide injected	I^{127} in thyroid as		Per cent of injected I^{131} recovered as	
		Diiodo-tyrosine	Thyroxine	Diiodo-tyrosine	Thyroxine
1*	γ	γ	γ		
		7.2	2.8	14.9	7.3
		6.8	2.9	12.8	4.9
		7.3	3.0	14.1	8.2
		6.8	2.7	8.7	3.2
		5.5	2.7	8.9	4.5
	100	5.1	2.7	0.16	0.019
		8.5	3.1	0.11	0.018
		6.8	2.7	0.18	0.029
		5.5	2.0	0.12	0.011
2†	5	5.4	2.4	7.1	2.7
		3.9	1.7	10.5	2.2
		6.6	2.7	11.1	2.6
		5.6	2.4	8.1	2.1
		5.9	2.7	7.5	2.5
	100	3.3	1.3	0.095	0.013
		3.2	1.3	0.045	0.007
		3.8	1.5	0.057	0.009
		7.0	2.9	0.048	0.008
		4.8	2.4	0.085	0.014
5†	10	2.8	1.0	5.4	2.2
		4.3	2.0	8.1	3.3
		5.4	2.8	11.4	4.5
		3.9	1.8	6.7	2.0
		6.6	2.4	11.8	4.0
	100	3.7	1.5	0.15	0.027
		3.2	1.1	0.061	0.013
		4.3	1.5	0.054	0.010
		3.5	1.8	0.041	0.007
		3.4	1.6	0.033	0.006
6†	10	3.0	1.5	8.5	3.2
		4.9	1.8	11.7	4.5
		7.1	2.8	15.7	5.8
		2.8	1.5	7.7	2.6
		5.6	2.5	11.3	4.4
	50	3.2	2.0	6.2	1.6
		3.4	1.8	2.5	0.48
		4.0	1.7	1.2	0.28
		3.1	1.5	0.82	0.24
	100	2.6	1.2	0.14	0.020
		4.9	2.4	0.088	0.014
		2.8	1.3	0.13	0.019
		3.6	1.5	0.096	0.012

* The rats of these experiments were sacrificed at 4.6 hours after the injection of labeled iodide.

† The rats of these experiments were sacrificed 4 hours after the injection of labeled iodide.

to the amount of I- turned over during the 4-hour period of observation,² we are safe in assuming that the specific activity of the plasma *inorganic iodide* throughout the 4-hour period of observation remained the same as that of the injected sample. We have therefore calculated the absolute amounts of iodide converted to diiodotyrosine in the gland during the 4 hours by multiplying the fraction of the injected

TABLE 3. AVERAGE VALUES* FOR 1) THE AMOUNT OF INJECTED I^{127} CONVERTED TO DIIODOTYROSINE AND 2) THE SPECIFIC ACTIVITIES OF DIIODOTYROSINE AND THYROXINE

Expt.	Iodide injected	Injected I^{127} converted to diiodotyrosine	S.A.† of diiodotyrosine iodine	S.A. of thyroxine iodine	S.A. thyroxine I	
					S.A. diiodotyrosine I	
1	5 100	0.60 0.14	1.8 0.022	2.1 0.007	1.2 0.32	
2	5 100	0.45 0.062	1.7 0.017	1.0 0.003	0.59 0.18	
5	10 100	0.87 0.068	1.9 0.019	1.7 0.008	0.90 0.42	
6	10 50 100	1.1 0.77 0.10	2.4 0.80 0.036	2.1 0.35 0.011	0.88 0.44 0.33	

* Each value is the average of 4 or 5 results obtained from as many rats (see Table 2)
 † Specific activity refers to the percentage of the injected I^{131} per unit weight of chemically measured iodine (I^{127}).

radioactivity recovered in the diiodotyrosine-like fraction of the gland by the gamma of I^{127} injected. For example, in experiment 2 (Tables 2 and 3), 9 per cent of the injected I^{131} was recovered in the diiodotyrosine fraction of the gland of the rats that were injected with 5 γ of I^{127} ; hence 0.09×5 or 0.45γ of the injected I^{127} had been incorporated into diiodotyrosine in the gland.

In Table 3 are recorded the average values obtained as described above for the diiodotyrosine synthesized by the thyroid glands of rats that were injected with either 5, 10, 50, or 100 γ of I^{127} as KI. It is clear that in each experiment the gland synthesized far more diiodotyrosine when 5 or 10 γ of I^{127} were injected than when 10 to 20 times these amounts of I^{127} were administered. These results therefore provide convincing evidence that excessive iodide exerts its inhibitory effects upon the reaction leading to the iodination of the phenolic group of tyrosine. This is in agreement with the *in vitro* observations of Morton *et al.* (1944) on surviving sheep-thyroid slices and with

* Since the diet contained 0.3 γ of iodine per gram, only negligible amounts of iodine could have been absorbed during the period of study. The rats were not fed during this period.

those of Li (1942), who showed from kinetic considerations that inorganic iodide inhibits the iodination of tyrosine in the test tube.

2. The Effect of Various Amounts of Injected Iodide Upon the Conversion of Diiodotyrosine to Thyroxine in the Thyroid Gland

The calculation made above for the injected I^{131} converted to diiodotyrosine during the 4-hour period of observation is valid only because the injected inorganic iodide is not appreciably diluted by the amounts of inorganic iodide present in the gland and plasma before the injection. The calculation for the amount of thyroxine synthesized is complicated by the fact that the newly formed diiodotyrosine mixes with a large amount of diiodotyrosine already present in the gland. For example, in experiment 1 the 0.6% of newly formed diiodotyrosine (i.e., during the 4 hours after the injection of labeled iodide) was diluted appreciably by the diiodotyrosine (Table 2). Hence a calculation similar to that described in the preceding section for the amount of injected labeled I^{127} converted to thyroxine in the gland would be meaningless as a measure of the *total amount* of thyroxine formed from diiodotyrosine during the interval studied. In other words, a calculation for the amount of injected I^{127} converted to thyroxine would provide a minimal value for the amount of diiodotyrosine converted to thyroxine, because it does not take into account the dilution of the newly formed diiodotyrosine by the diiodotyrosine already present in the gland.

Hevesy (1938) has pointed out that the ratio,

$$\frac{\text{specific activity of compound A}}{\text{specific activity of the precursor of compound A}}$$

can be used to compare the turnover of compound A in a single tissue under 2 different conditions. Many factors may influence the specific activity of the compound or that of its precursor, but the ratio of their specific activities is independent of changes in permeability, distribution of the labeling agent, etc., and varies only with the turnover rate of compound A. A recent application of the use of relative specific activities as an index of turnover was made by Zilversmit *et al.* (1948).

We have made use of relative specific activities

$$\left(\text{in this case, } \frac{\text{specific activity of thyroxine}}{\text{specific activity of diiodotyrosine}} \right)$$

to compare the conversion of diiodotyrosine to thyroxine under the 2 experimental conditions studied here, namely high- and low-iodide injections. The values for the ratios are recorded in Table 3. If the rate of conversion of diiodotyrosine to thyroxine had remained the same under both conditions, then this ratio would also have remained the same in the presence of low- and high-iodine injections. Table 3 shows, however, that a 2-to-4-fold decrease in the ratio occurred when

the amount of I^{127} was increased from 5 or 10 γ to 100 γ . This indicates that the conversion of diiodotyrosine to thyroxine was considerably slower after the injection of the high amounts of iodine. Whether this is due to a specific action of the excess iodide on this step or whether the interference in the conversion of diiodotyrosine to thyroxine results simply from the smaller amounts of diiodotyrosine formed can not be stated with certainty.

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SUMMARY

The effects of the introduction of excessive amounts of inorganic iodide upon 1) the incorporation of iodide into diiodotyrosine and 2) the conversion of diiodotyrosine to thyroxine in the thyroid gland were investigated with the aid of I^{131} .

Since the amounts of inorganic iodide injected were large with respect to those present in the gland and plasma before the injection, it was possible to calculate the amounts of newly formed diiodotyrosine in the gland from the proportions of the injected I^{131} incorporated into this fraction. Far more diiodotyrosine was synthesized by the rat thyroid when 5 or 10 γ of iodide were injected than when 10 or 20 times these amounts were administered.

In order to compare the conversion of diiodotyrosine to thyroxine in rats that received high- and low-iodide injections, use was made of the ratio of the specific activity of thyroxine to that of its precursor, namely diiodotyrosine. A 2-to-4-fold decrease in this ratio was observed when the amount of I^- injected was increased from 5 or 10 γ to 100 γ . This indicates that the conversion of diiodotyrosine to thyroxine was considerably slower when the higher amounts of iodine were injected.

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THE NATURE OF THE ANEMIA OF PREGNANCY IN THE RAT¹

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ALTHOUGH the anemia of pregnancy in the rat was once thought to be attributed to nutritional deficiencies, some experiments (Mitchell and Miller, 1931; Beard and Myers, 1933; Van Donk, Feldmen and Steenbock, 1934) demonstrated the inadequacy of dietary supplements in the prevention of this condition. The importance of the placenta in the development and maintenance of pregnancy anemia in the rat was demonstrated and the possibility of an endocrine basis for the anemia was suggested (Newcomer, 1947). In none of these experiments was the exact nature of the anemia described, although hemodilution was suggested because erythrocyte changes paralleled hemoglobin changes. In this investigation the nature of absolute and relative changes in some of the blood constituents during pregnancy in the rat was determined. Studies were made on the number of erythrocytes, hemoglobin concentration, hematocrit, whole blood and plasma specific gravities, and blood volume at three stages of pregnancy in rats.

MATERIALS AND METHODS

Long-Evans adult, nulliparous, female rats weighing 169-341 grams were used. Pregnancies were dated from the first day spermatozoa were observed in vaginal smears taken before 9 A.M.

Blood samples of 0.2 or 0.5 cc. were obtained from the heart while keeping the rats under light ether anesthesia and using heparin to prevent clotting. The determinations were made in the following order from the 0.5 cc. sample: erythrocyte count, whole blood specific gravity, hematocrit, and hemoglobin concentration. The remaining blood sample was centrifuged for 30 minutes under a relative centrifugal force (R.C.F.) of 1065, and the specific gravity of the plasma was determined. All specific gravity measurements were made in duplicate using the LaMotte Falling Drop Densimeter. Erythrocyte counts were made with a Spencer Bright-Line Haemocytometer and hemoglobin concentrations were measured by the acid hematin method using a Fisher Electro-Hemometer. Color indices were calculated according to the formula:

$$\frac{\text{gm. Hb/100cc. experimental rat blood}}{\text{gm. Hb/100cc. normal rat blood}} \text{ divided by } \frac{\text{experimental RBC count}}{\text{normal RBC count}}$$

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(Magner, 1938). Hematocrit readings were made in Van Allen tubes after centrifugation (R.C.F. of 1065) for 30 minutes.

Blood volumes were determined according to a slight modification of the method of Griffith and Campbell (1937). To standardize the dye, a saturated stock solution (5%) of vital red (Trypan red) dye was prepared using 0.9% saline. The solution was filtered and a series of dilutions from 1:1000 to 1:10,000 was prepared. The color intensities were read in a Klett-Summerson photoelectric colorimeter using a 540m μ filter. When colorimeter scale readings (ordinates) were plotted against the reciprocals of the dilutions (abscissas), a straight line was obtained. The standardization of the stock solution was carried out in triplicate over a range of 9 different dilutions.

In making a blood volume determination, 0.36 cc. of the stock solution were injected intravenously into an ether-anesthetized rat. After 4.5 minutes, 0.2 cc. of blood were removed from the heart, added to 11.8 cc. of saline, and centrifuged (R.C.F. of 1065) for 20 minutes. The intensity of the resulting pink solution was determined in the colorimeter. Since the dilution of the sample is equal to 1:60 and that of the dye is equal to 0.36 cc./blood volume, the total dilution of the dye is the product of the dilution of the sample times the dilution of the blood. Total dilution, therefore, is equal to blood volume times 60/0.36, or blood volume is equal to 0.006 times the total dilution. The reciprocal of the total dilution was read on the standard curve, and the blood volume equaled 0.006/abscissa reading. Since 0.36 cc. of dye was injected, this amount may be subtracted from the calculated volume to correct for the volume of dye added to the blood.

Statistical significances of the data were calculated according to *t*-methods as described in Snedecor (1946; chapters 2 and 4).

All measurements with the exception of blood volume were recorded on the 1st, 13th, and 21st days of pregnancy in one group of rats. In order to reduce any possible effect on hemopoiesis resulting from the sampling of blood, a second group of rats was studied on the 1st and 22nd days of pregnancy (also 2nd day post-partum) and only 0.2 cc. of blood were removed each time. In this group, determinations were made only on erythrocyte count and hemoglobin concentration. In a third group, blood volumes were measured on the 1st and 22nd days of pregnancy and on the 2nd day post-partum the 1st and 14th days of pregnancy and on the 2nd day post-partum in others. Control observations were made on non-bred females in each of the three groups. In the controls of the first group, however, only plasma specific gravities were determined.

OBSERVATIONS AND RESULTS

The decrease in the erythrocyte number and hemoglobin concentration during normal pregnancy (Tables 1 and 2) agreed with the results of other experiments (Van Donk, Feldman and Steenbock, 1934; Neweomer, 1947). Color index calculations demonstrated the parallel nature of the changes in erythrocyte count and hemoglobin concentration. Although there was a significant increase in the color index in the rats of the first group, all values remained within the limits of normal human blood which are 0.85-1.15 (Beek, 1938). The hematocrit readings were lower by the 13th day, but only

significantly so by the 21st day of pregnancy (Table 1).

There was a significant decrease in whole blood specific gravity during the latter half of pregnancy, but more important, a significant decrease in plasma specific gravity (Table 1). To determine if any change in plasma specific gravity would occur in a group of non-bred animals, 6 nulliparous females were studied. The mean specific gravity was 1.0272 ± 0.0007 on the 1st day and 1.0270 ± 0.0003 on the 13th

TABLE 1. OBSERVATIONS ON THE BLOOD OF FEMALE RATS AT THREE STAGES OF PREGNANCY

	1st day	13th-14th day	21st-22nd day ¹
Body weight (gm.)	200 ± 8^2	230 ± 8	261 ± 9
Erythrocytes (million/cmm.)	8.84 ± 0.21	8.04 ± 0.15	7.62 ± 0.19
Hemoglobin (gm./100 cc.)	14.2 ± 0.2	13.8 ± 0.4	13.2 ± 0.2
Color index	0.99 ± 0.02	1.05 ± 0.02	1.07 ± 0.02
Hematoerit (%)	42.9 ± 1.3	40.0 ± 1.2	38.6 ± 0.6
Specific gravity whole blood	1.0573 ± 0.0004	1.0566 ± 0.0007	1.0541 ± 0.0003
Specific gravity plasma	1.0280 ± 0.0004	1.0284 ± 0.0004	1.0266 ± 0.0004
Blood volume ³ (cc.)	17.34 ± 0.55	20.99 ± 1.09 (5)	24.45 ± 0.72
BV/100 gm. body wt.	8.2	8.0	8.2

¹ Values of P for the determinations at the 21st-22nd day stage are less than .01 except for color index and plasma specific gravity where $P = .02$.

² Mean value and standard error; each figure represents determinations on 9 animals except where the number appears in parentheses.

³ Blood volume from rats of Group 3 in which only blood volumes were determined. The average body weights on days 1, 14, and 22 of pregnancy were 212 ± 6 , 260 ± 6 and 299 ± 8 gm. respectively.

day. On the 21st day it had risen to 1.0297 ± 0.0003 , a significant increase over the previous values (P less than .01). At no other time in these experiments were specific gravities of this high magnitude recorded. In view of this increase in specific gravity obtained in non-bred rats, another group of 6 rats was studied in which samples were taken on the 1st, 15th and 29th days. The mean values were 1.0272 ± 0.0004 , 1.0275 ± 0.0001 and 1.0285 ± 0.0004 respectively. This increase was not statistically significant (P between .1 and .05).

Studies revealed a marked increase in blood volume during pregnancy, but no increase was apparent when the results were calculated on the basis of the increased body weight (Table 1). The blood volume of 11 control females averaging 222 ± 11 gm. in weight was 17.23 ± 0.76 cc. on the 1st day. After 21 days the mean body weight increased to 243 ± 8 gm. and the blood volume increased to 18.39 ± 0.90 cc. The

ratio of blood volume to body weight remained the same on the 1st and 22nd days (7.8 cc./100 gm. and 7.6 cc./100 gm.). The increase in blood volume due to pregnancy was 6 times greater than the increase in the non-bred controls.

When the mean total number of erythrocytes and grams of hemoglobin within the animal were calculated, the results showed an

TABLE 2. OBSERVATIONS ON THE BLOOD OF FEMALE RATS AT TWO STAGES OF PREGNANCY AND 2ND DAY POST-PARTUM (GROUP 2)

	1st day	22nd day ¹	2nd day post-partum
Body weight (gm.) pregnant rats non-bred controls	248 ± 9 ² 208 ± 18	351 ± 10 231 ± 15	294 ± 8 —
Erythrocytes (million/cmm.) pregnant rats non-bred controls	8.83 ± 0.16 8.13 ± 0.22	7.25 ± 0.30 8.22 ± 0.32	7.32 ± 0.53 —
Hemoglobin (gm./100 cc.) pregnant rats non-bred controls	14.4 ± 0.2 13.9 ± 0.2	12.2 ± 0.3 13.7 ± 0.2	12.2 ± 0.7 —
Color index pregnant rats non-bred controls	1.01 ± 0.02 1.05 ± 0.02	1.04 ± 0.03 1.03 ± 0.02	1.03 ± 0.05 —
Blood volume ³ (cc.) pregnant rats	—	—	23.36 ± 1.36
BV/100 gm. body weight	—	—	8.1

¹ Values of P for erythrocyte and hemoglobin determinations in the pregnant animals on the 22nd day are less than .01. The color index was not significantly increased (P greater than .5). No significant changes in control determinations.

² Mean value and standard error; each figure represents determinations on 4 or more animals.

³ Blood volumes determined on rats from colony and Group 3. The average body weight was 286 ± 12 gm.

actual increase in these constituents during pregnancy. Furthermore, the increase was 2-3 times greater than that observed in non-bred females over a similar period (Table 3).

A few observations on the condition of the blood on the 2nd day post-partum were made. The mean erythrocyte and hemoglobin values showed little change from the mean values on the 22nd day of pregnancy, but large individual variations were encountered (Table 2). The blood volume bore the same relationship to the body weight as had been observed in the other experiments. The calculated total amount of hemoglobin and erythrocytes was less than on the 22nd day of pregnancy and slightly less than on the 1st day (Table 3). No attempt was made to determine the loss of blood at parturition which might influence the general blood picture at this time.

TABLE 3. CALCULATED MEAN TOTAL NUMBER OF ERYTHROCYTES AND GRAMS OF HEMOGLOBIN IN FEMALE RATS AT THREE STAGES OF PREGNANCY AND 2ND DAY POST-PARTUM

	1st day	13th-14th day	21st-22nd day	2nd day post-partum
Group 1				
Pregnant rats				
Total RBC ¹	14.5×10^{10}	14.9×10^{10}	16.3×10^{10}	—
Total Hb ¹	2.32	2.54	2.80	—
Group 2				
Pregnant rats				
Total RBC	18.0×10^{10}	—	20.8×10^{10}	17.4×10^{10}
Total Hb	2.93	—	3.51	2.90
Non-bred controls				
Total RBC	14.0×10^{10}	—	15.1×10^{10}	—
Total Hb	2.40	—	2.52	—

¹ Differences in body weights between groups of rats in which erythrocyte and hemoglobin determinations were made and groups in which blood volumes were recorded were adjusted through use of the blood volume/body weight ratio

DISCUSSION

From the experiments reported here, it seems that the anemia of pregnancy in the rat is the result of hemodilution. The basic evidence for this was (1) the decrease in plasma specific gravity and (2) the increase in blood volume which remained constant relative to body weight. The ordinary methods of determining anemia from erythrocyte count and hemoglobin concentration do not indicate the nature of this anemia.

When plasma specific gravity determinations were made on nulliparous rats, there was an increase at the same time the pregnant animals showed a decrease. Whatever the cause of the increase in plasma specific gravity in the non-bred animals, it would seem likely that a similar mechanism would work in the pregnant rats. The decrease in plasma specific gravity during pregnancy occurred in spite of any such mechanism.

Although blood volume increased in the rat during pregnancy, it remained constant on the basis of 100 gm. of body weight. This was also true in the non-bred controls in which the weight gain was not as great. It has been shown that the body weight of the pregnant rat, not including the weight of the growing conceptus, increased more rapidly than normal (Newton, 1935), yet in these experiments the blood volume change paralleled the change in total weight of which most was contributed by the increase in the uterus and its contents.

The post-partum studies, although few in number, showed that there was a rapid loss in blood volume after parturition. There was, however, no appreciably change in erythrocyte and hemoglobin levels from those of the 22nd day of pregnancy. Since no measurement of

the loss of blood in parturition was made, it was difficult to interpret the post-partum changes in the blood.

When calculations were made on the change in the total quantity of erythrocytes and hemoglobin from the 1st to the 22nd day of pregnancy, it was observed that an absolute increase in the number of cells and the amount of hemoglobin occurred. The anemia of pregnancy is more apparent than real. It would appear that during the gestation period an increase in both cells and blood volume occurred, but the blood volume increased to a greater degree.

The nature of the anemia of pregnancy in the rat corresponds in many respects to the type in the human classified as "physiologic" (for review of recent literature see Bethell, Sturgis, Rundles and Myers, 1945). A physiological basis for the development of a hydremia in the human during the gestation period was presented by Dieckmann and Wegner (1934), and it seems probable that their explanation might be extended to cover the rat. They believe that during pregnancy there is a rapid expansion in the area of the circulatory bed and fluid must be added to the blood to maintain an adequate circulatory volume. This larger volume of blood has a lower specific gravity and, hence, a lower viscosity. The lowering of the viscosity of the blood reduces the burden on the heart resulting from the increase in the circulatory volume. Thus, the fundamental process is a shift in water balance to protect the heart.

SUMMARY

Studies were made on the erythrocyte count, hemoglobin concentration, hematocrit, whole blood and plasma specific gravities, and blood volume of rats at three stages of pregnancy and on the 2nd day post-partum. It was observed that a significant decrease in whole blood and plasma specific gravities occurred in the pregnant rats, but an increase in plasma specific gravity occurred in the non-bred controls. The total blood volume increased during pregnancy, but the ratio of total blood volume to body weight increased only slightly in pregnant rats and blood volume remained constant in non-bred controls, the body weight and blood volume increased only to a slight extent and the blood volume/body weight ratio remained constant. Calculations of total circulating erythrocytes and hemoglobin in pregnant rats indicated that in both of these elements a sharp rise occurred which was proportionally greater than in the non-bred controls. From this evidence, it appears that the anemia of pregnancy in the rat is due to hemodilution in which blood volume increases proportionally faster than the erythrocyte number.

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NOTES AND COMMENTS

HISTOLOGICAL EFFECT OF THIOURACIL ON THE FETAL THYROID OF THE MOUSE¹

THE PURPOSE of this work was to ascertain the time at which the fetal thyroid of the mouse begins secretory activity, and to determine the effect of thiouracil upon the growth and development of the fetal thyroid.

Up to the present time, no study has been undertaken to determine when the fetal thyroid of the mouse begins to function. With the discovery of goitrogenic substances which are known to inhibit thyroxine secretion, it seemed of interest to determine to what extent the fetal thyroid of the mouse might be influenced by the administration of a goitrogenic chemical such as thiouracil (Astwood, 1943; MacKenzie & MacKenzie, 1943; Franklin, Lerner, & Chaikoff, 1944.)

EXPERIMENTAL PROCEDURE

Thiouracil² was administered in the feed (0.2%) to pregnant female mice from the time of conception to the time of sacrifice. These females were sacrificed daily along with controls from the twelfth day of pregnancy until the time of parturition. After sacrifice, the thyroids were fixed in Bouin's solution and imbedded in paraffin. They were then sectioned at five to six microns and stained with hematoxylin-eosin. The fetuses were taken from the uteri of the mothers, and their tracheae with thyroids attached were removed. The above fixing and staining technique was again employed.

In an effort to obtain timed embryos, the following procedure was used: The males were put in with the females at about ten P.M., and were removed at approximately eight A.M. the following morning. Vaginal plugs in the females were used as an indication of copulatory activity during the night. The females were sacrificed at about eight A.M., and the age of the fetus was calculated from eight in the morning. That is, the embryo was considered to be one day old 24 hours after the males were removed. The majority of the females gave birth between 20½ and 21 days after mating.

RESULTS

The thyroids of the mothers kept on the control ration were normal in appearance. They contained well defined follicles whose epithelial cells were of normal cuboidal shape. The lumina of the follicles contained deeply staining colloid. In contrast to this, the thyroids of the females fed thiouracil showed ill-defined follicles. The cuboidal cells had increased in size and had become columnar. No colloid could be found in the follicles. Macroscopically, the glands were much enlarged and weighed from four to five times as much as the glands of the females on the normal ration.

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¹ Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 1113.

² Thiouracil was kindly supplied by the Lederle Laboratory of Pearl River, N. Y.

Examination of the normal fetal thyroids histologically showed slight follicle and colloid formation at the sixteenth day. More colloid and clearly defined follicles became apparent as the fetuses progressed toward term. The greatest amount appeared in those fetuses that were sacrificed immediately after parturition.

Examination of the fetal thyroids of the experimental group failed to reveal any colloid. The follicles were not at all clearly defined, as the cells were arranged in a rather haphazard manner. These cells were slightly enlarged. As the fetuses approached term, still no colloid could be seen in the follicles and their general appearance resembled that of the maternal thyroid of an animal fed thiouracil. This was also true of the experimental mice that were sacrificed immediately after birth.

From these observations it was concluded that when the mouse fetus is subjected to the influence of thiouracil, thyroid follicles and colloid formation is suppressed.

DISCUSSION

These data on the development of the mouse thyroid indicate that follicle formation occurs on the 16th day. This agrees with the observations of Hall & Kaan (1942) in the rat, but is somewhat earlier than that reported by Gorbman & Evans (1941) and Kull (1926). It would appear that the time of thyroid development in the two species is approximately the same.

Moody (1910) reported that colloid is found in the pig's thyroid early in embryonic life, when the fetus is only 45 mm. long. Follicles do not appear until the embryo is 70 mm. long, that is at approximately 50-55 days of age.

Fenger (1912) stated that the active thyroid principle is present in the thyroid of fetal calves 6-12 weeks of age.

In an earlier report, Fenger (1912) determined iodine in the thyroid of fetal sheep 2-4 months of age. He believed that the thyroids were active at that time.

Abbott and Pendergast (1937) reported that follicles were found as early as the second month of intrauterine life in calves.

Our work confirms the previous reports by Hughes (1944) Schultze and Turner (1945), and Goldsmith, Gordon, & Charipper (1945) that thiouracil fed to mothers will cause an enlargement of the fetal thyroids. The mechanism by which thiouracil affects the fetal thyroid would involve the passage of either thiouracil or thyrotrophic hormone or a combination of the two across the placenta.

The work of Friesleben and Kjerulf-Jensen (1946) indicates that in the rat thiouracil will pass across the placenta.

Tobin (1941) found that the fetal thyroids of rats were not stimulated by thyrotrophin injected into the mother. He concluded that thyrotrophin does not pass through the placenta as it is a very large protein molecule. This suggests that the fetal pituitary of mice may begin the secretion of the thyrotrophin on or before the 16th day.

Schultze and Turner (1945) reported that thyroids of fetal goats past mid-term of intrauterine life were greatly enlarged when the mothers were treated with thiouracil or thiourea. Prior to mid-term thiouracil had no effect on the fetal thyroid.

SUMMARY

Evidence has been presented which indicates that in the mouse thyroid follicle and colloid formation begin on the 16th day of fetal life. Feeding 0.2% thiouracil in the feed of the mother was observed to inhibit the formation of thyroid follicles and depressed colloid formation in the fetus.

It is believed that this effect is due to the passage of thiouracil through the placenta.

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THE IMPORTANCE OF TYPE OF ZINC USED IN Zn-HCl HYDROLYSIS OF URINE

In 1937 it was reported that boiling of urine under a reflux for 3 hours with 4 gm. % of zinc dust and 15 volumes % of concentrated HCl resulted in a marked increase in the yield of benzene or ether soluble estrogens over that acquired by the optimum conditions of simple acid hydrolysis (10 minutes with 15 vol. % of HCl). Although it was demonstrated that conversion of estrone into a more active estrogen contributed to this increase in potency and that more complete hydrolysis with less destruction might also account for a part of it in certain urines, further investigation revealed that the greatest relative increases were from urines in which neither of these factors could play any significant part. From these studies together with the results acquired from comparing the total activity after simple HCl hydrolysis (Tzn) with the sum of the separated estrogens after simple HCl hydrolysis (To) in women under various physiological, pathological and experimental conditions, it was concluded that the major source of this additional estrogenic activity was some estrogenically inactive oxidation product of the estrogens that was rendered estrogenic by Zn-HCl hydrolysis. The additional potency

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after Zn-HCl hydrolysis, therefore, that is not accounted for by estrogens excreted as such has been interpreted in this laboratory as providing a gauge of the rate of oxidative inactivation of estrogens within the body.

It has been reported to us, however, that other investigators have been unable to get consistent results from Zn-HCl hydrolysis and the clue to their failure has only recently been revealed to us. When the effect of Zn-HCl hydrolysis was first observed we were using a preparation of powdered zinc put out by Merek and labeled "technical." When this was used up we had some trouble getting another batch that had the same effect, but finally found that Merek's Zn dust, Reagent, was satisfactory and procured a large batch of this that lasted until December, 1946. A new batch procured from Merek at this time, although bearing the same label, gave inconsistent and confusing results. During the process of refluxing with Zn and HCl, in the specified proportions, the urine should become practically colorless and clear with a large "glob" of zinc floating through the solution and with profuse evolution of hydrogen throughout the 3 hour period. At the end of the 3 hour period of refluxing neither the zinc nor the acid should be used up, there being plenty of unchanged zinc left and the titratable acidity

TABLE 1

Specimen No.	Total activity after simple HCl hydrolysis (15 vol. %) r.u./24%	Total activity after various Zn-HCl hydrolyses r.u./24 hrs.				
		Merek's reagent Zn-dust			Mallinekradt Zn-dust, A.R.	
		4 gm. % Zn + 15 vol. % HCl	More Zn added	More Zn + HCl added	4 gm. % Zn + 15 Vol. % HCl	
					#8680	S.H.G. #8681
1 (urine)	92	140				380
2 (urine)	55	132				220
3 (urine)	230	480				2,000
4 (urine)	250	670				2,100
5 (urine)	490	300				2,000
6 (urine)	27,000	13,000	1,000		2,000	40,000
7 (urine)	540	2,000	670		2,000	2,500
8 (urine)	470	1,000		1,000	2,000	2,800
9 (urine)	292	800		1,000	1,500	2,000
10 (Estrone)*	80	200		<100	400	500

* 40 micrograms of crystalline estrone in 100 cc. concentrated phosphate buffer solution (see text).

having been reduced from 1.5 N to approximately 1.3 N. With this 1946 batch of Merek's Zn dust (and with most other preparations since tried) the zinc and/or the acid were used up after about 1 hour of refluxing, the urine turned yellow to red, depending upon the specimen, and either became cloudy with a thick emulsion or contained a white powder that settled out upon cooling. The addition of more acid would clear the solution and of more zinc would again decolorize it but, as shown in Table 1, neither of these procedures would correct the marked loss of estrogenic activity that was apparent when a good batch of zinc dust was finally acquired. We have tried adding zinc oxide, iron powder and small amounts of mercury to the Merek Reagent Zn dust with no better results.

We are indebted to Mr. N. D. Barry of the Mallinekradt Chemical Works for providing us with several preparations of zinc, one of which was found to give the desired results, viz., Zn dust S. H. G. (as it was labelled in the sample originally provided by him) or Mallinekradt Zn dust, Analytical Reagent, #8681. As is seen in Table 1 another batch of Mallinekradt Zinc dust, A. R., #8680, gave practically the same results but a slightly lower

yield in 3 urine specimens, all three of which turned yellow during hydrolysis with this batch but not with #8681. According to Mr. Barry the difference between batches lies in the method of processing, not in the chemical composition or impurities.

In the original publication it was reported that the estrogenic activity of crystalline estrone was increased 5 to 7 fold by Zn-HCl hydrolysis. As shown in Table 1, specimen 10, this provides an excellent test for the particular zinc dust that is being used. The estrone must be in a buffer solution of high salt content (69 mg. $\text{Na H}_2 \text{PO}_4 \cdot \text{H}_2\text{O}$ plus 179 gm. $\text{Na}_2 \text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ per liter) and in a concentration of no greater than 50 micrograms per 100 cc. From our early observations we believe that estrone is converted into a mixture of α and β estradiol.

The use of this Zn-HCl hydrolysis is applicable to investigations in which the separated estrogens after simple HCl hydrolysis are also being studied. The increase that is not accountable to estrogens excreted as such and is presumably derived from estrogen oxidation products may be calculated by subtracting from the Tzn value the sum of the activities of estrone $\times 6$ plus estradiol plus estriol. Despite the fact that these calculations introduce the possibility of considerable error, results so acquired have yielded information of considerable interest and apparent significance.

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ASSOCIATION AWARDS FOR 1949

THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb & Sons Award of \$1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner; 1941—Dr. Philip E. Smith; 1942—Dr. Fred C. Koch; 1943—no award was given; 1944—Dr. E. A. Doisy; 1945—Dr. E. C. Kendall; 1946—Dr. Carl G. Hartman; 1947—Drs. Carl F. and Gerty T. Cori; 1948—Dr. Fuller Albright. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russel; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl G. Heller. The work cited may be either in the field of preclinical or clinical endocrinology. The Award is for \$1,200.00. If within 24 months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, the Award will be increased to \$1,800.00.

THE AYERST, McKENNA & HARRISON FELLOWSHIP

The Ayerst, McKenna & Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin, and in 1948 to Dr. Ernest M. Brown, Jr. This Fellowship is designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. The Fellowship may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend for the Fellowship will vary in accordance with the qualifications of the appointee, but will not exceed \$2,500.00. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. The nominee must present evidence or scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals; submit a program of proposed study; indicate one or more institutions where the proposed program shall be carried out; submit statements of approval from the investigators with whom he proposes to conduct his research.

Each member has the privilege of making one nomination for each award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to endocrinology and by a bibliography of the nominee's most important publications, and reprints, if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 15, 1949.

ERRATUM NOTICE

Inadvertently the figure below was omitted from Dr. Marlow's article entitled "16-Ketoestrone in the Kober Reaction" which appeared on page 479 of the June issue of *Endocrinology*.

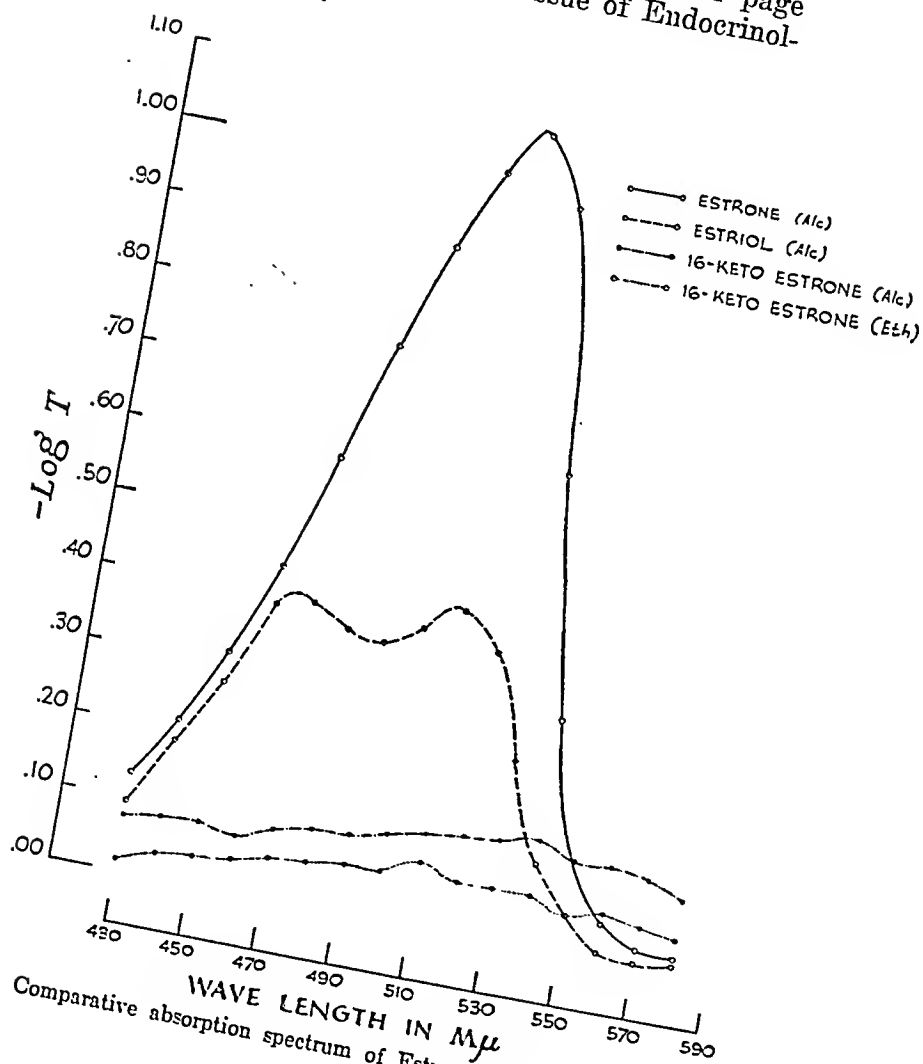


Fig. 1. Comparative absorption spectrum of Estrone, Estriol and 16-Ketoestrone.

that thyroxine in an optimal dose increased significantly the percentage of castrate female mice which responded with mammary lobule-alveolar development to minimal doses of progesterone and estrone. Mixner (1947), in work with mice concluded that thiouracil acts in a manner similar to thyroidectomy in decreasing the responsiveness of the mammary gland to exogenous mammary growth stimulating substances.

Reeder and Leonard (1944), reported that adrenalectomy in normal or castrate immature male rats resulted in an increased number of lateral buds on the duct system of the mammary tree, and in some cases, increased end bud growth. Administration of estrogen caused dilatation of the lateral buds. On the other hand, Trentin and Turner (1947), reported that adrenalectomy in castrate male rats resulted in thin atrophic ducts and complete elimination of lobule-alveolar development. Although administration of estrogen caused noticeable duct stimulation, it was relatively ineffective in stimulation of alveolar development. Cowie and Folley (1947), reported that regressive changes were frequently, but not invariably, observed following adrenalectomy; increased arborization of the duct system was never observed.

METHODS

Seventy-two albino rats were castrated at three weeks of age and divided into eight groups. They were fed a ration for laboratory animals *ad libitum*, and maintained at constant temperature (76 F.) and humidity. The experimental techniques included adrenalectomy and treatment with thiouracil and estrogen, alone or in various combinations. The litters were divided so that comparison on the basis of estrogen treatment could be made between litter mates. All of the experimental techniques for different groups were performed when the rats were at the same age.

The adrenalectomized rats were given one per cent NaCl in the drinking water. Thiouracil¹ was administered at the rate of 0.1 per cent in the feed for 45 days. Diethylstilbestrol² was administered subcutaneously in an oil base at the rate of 10 gamma daily during the last ten days of the experimental period. The rats were destroyed on the eleventh day. The right abdominal mammary gland was removed and stained *in toto* with Harris's hematoxylin. Body weighings were made at the beginning and end of the experimental periods. Examination was made at autopsy for completeness of adrenalectomy and for presence of accessory cortical tissue.

RESULTS

The effects of estrogen on mammary structure of castrate rats

Each of two litters comprising 16 rats was equally divided between groups (1) and (2). These rats were castrated at three weeks of age,

¹ The thiouracil was supplied by Dr. Stanton L. Hardy, Lederle Laboratories, Pearl River, New York.

² The diethylstilbestrol was supplied by Dr. D. F. Green, Merck and Co., Rahway, New Jersey.

then placed in experimental cages and maintained on regular rations for 65 days. During the last ten days group (2) received 10 gamma of diethylstilbestrol subcutaneously in an oil base daily. On the day following the experimental period, both groups were killed and the right abdominal mammary gland was removed. At autopsy the controls showed an average gain of 117 grams and the estrogen injected 109 grams during the last 45 days of the experiment.

All of the mammary glands of the control group showed a uniformly bare duct system with no indication of any lobule-alveolar development. This is the typical picture that is found in the mammary glands of castrated rats. Examination of the mammary glands of the rats injected with estrogen showed extension of the duct system and distinct end bud growth. In some cases definite lobule-alveolar growth was present. These glands showed a typical response to estrogen administered in greater than threshold amounts.

The effects of estrogen on mammary structure of thiouracil treated castrate rats

Each of two litters comprising 18 rats was equally divided between groups (3) and (4). These rats were castrated at three weeks of age. Both groups were placed in experimental cages at seven weeks of age and fed the regular ration to which had been added 0.1 per cent thiouracil. They were maintained on this ration for a 45 day period, the last ten of which group (4) received 10 gamma diethylstilbestrol daily. Both groups were destroyed on the eleventh day and the mammary glands removed. The controls gained an average of 61 grams and the estrogen treated gained an average of 45 grams during the experimental period.

The mammary glands of the control group showed a shortened and thickened duct system. The mammary glands of two rats showed a small amount of lobule-alveolar development. The mammary glands of the estrogen injected rats showed a considerable amount of lobule-alveolar development, especially toward the ends of the ducts. The ducts were shortened and thickened, and the lobule-alveolar growth was much more dense than that of the estrogen treated rats which did not receive thiouracil (group 2). In comparison with the castrate rats which did not receive thiouracil (group 1), the mammary glands of group (3) showed shorter and thicker ducts.

The effects of estrogen on mammary structure of adrenalectomized castrate rats

Each of two litters comprising 20 rats was equally divided between groups (5) and (6). These rats were castrated at three weeks of age. All were adrenalectomized at five weeks of age, and placed on one per cent NaCl in the drinking water. A period of ten days was allowed for recovery from the effects of the operation, at which time they were

placed in the experimental cages and fed regular rat ration for a period of 45 days. During the last ten days group (6) received 10 gamma of diethylstilbestrol daily. All were destroyed on the eleventh day and the mammary glands removed. The control group gained an average of 86 grams while the estrogen treated group gained an average of 79 grams during the experimental period.

The mammary glands from the control group had long atrophic ducts with no lobule-alveolar development. The ducts were longer and thinner than those of any of the groups which did not receive estrogen. The mammary glands of the rats which received estrogen were also very extensive in area and were greatly thickened grossly as a result of extensive lobule-alveolar development. The over-all development of these glands was somewhat greater than that of the estrogen treated castrated rats.

The effects of estrogen on mammary structure of thiouracil treated adrenalectomized castrate rats

Each of two litters comprising 18 rats was equally divided between groups (7) and (8). These rats were castrated at three weeks of age. They were adrenalectomized at five weeks of age and placed on one per cent NaCl in the drinking water. After a ten day recovery period they were placed in experimental cages and fed the regular rat ration to which 0.1 per cent thiouracil had been added. They were maintained on this ration for 45 days the last 10 of which group (8) received 10 gamma of diethylstilbestrol daily. The rats were destroyed on the eleventh day and the mammary glands removed. The average weight gain for the controls was 50 grams and that of the estrogen treated was 37 grams for the experimental period.

The mammary glands of the control group showed very short atrophic ducts. The ducts of these glands were shorter than those of any other group and were thinner than those of either the castrate or the thiouracil treated castrate group. The mammary glands of the estrogen treated group showed a short, thick duct system with considerable lobule-alveolar development. The peripheral alveoli were especially dilated. The over-all development of these glands was greater than that of any other group.

DISCUSSION

The purpose of the present work was to determine the effects of estrogen on the mammary structure of thiouracil treated, adrenalectomized castrate rats. In order to evaluate the factors responsible for the mammary growth obtained in these rats, it was necessary to set up a series of experiments which would demonstrate the effects of various combinations of treatments. In addition, these control groups served to confirm certain results of earlier workers in similar experiments. The mammary structure of castrate and estrogen treated

castrate rats is sufficiently well known to need no further confirmation (Turner and Schultze, 1931; Nelson 1936).

It is also well known that the mammogenic hormones of the pituitary gland are a factor in the response of the mammary gland to estrogen injection. In the present work however, the pituitary gland can be considered merely as one of the many factors operating in the body which affect the mammary gland; i.e., the specific action of the mammogenic hormones on mammary growth need not be considered in these experiments. The response of the mammary gland of this strain of rats to castration and subsequent injection of estrogen was typical, as previously described in this paper. Castration reduced the mammary gland to a bare duct system. In addition to duct extension, the amount of estrogen injected also caused some lobule-alveolar development.

The results of thiouracil treatment are essentially in agreement with the work of Smithcors (1945) and Smithcors and Leonard (1942). In general the administration of thiouracil inhibited extension of the duct system. Subsequent injection of estrogen resulted in marked lobule-alveolar development without appreciable further duct extension. However, it has been shown that in the mouse (Mixner and Turner, 1942; Mixner, 1947), thyroidectomy or thiouracil administration decreases the mammary growth response to estrogen and that thyroxine in optimal doses increases the ability to respond with lobule-alveolar development. This difference may be attributed to the fact that the mouse is normally hypothyroid, whereas the rat is normally hyperthyroid.³ It has been shown that rats respond to minimal doses of thyroxine by retardation of growth, while mice on the same dosage increase their growth rate (Meyer and Wertz, 1938a; Koger and Turner, 1943). Minimal doses of thiourea increase the growth rate of the rat (Astwood and Bissell, 1944).

In Smithcors' work it was suggested that failure of thiouracil to induce the changes following thyroidectomy might be due to the relatively short period of treatment (18-35 days). In the present work the mammary glands of the thiouracil treated rats more closely resembled those following thyroidectomy. This may be due to longer periods of treatment (45 days) in the present work. The shortened and thickened ducts found following thiouracil treatment may result from the modification of the specific action of the pituitary mammogenic or other general growth stimulators in the hypothyroid state.

The results of estrogen administration in the thiouracil treated rats in the present work confirm the results obtained by Smithcors (1945), and are in agreement with the results of Smithcors and Leonard (1942) and Leonard and Reece (1941).

³ An additional paper (Trentin, Hurst, and Turner 1948) has appeared since the writing of this paper. In the rat, but not in the mouse, thiouracil augmented the mammary growth response to estrogen.

The increased lobule-alveolar development obtained following estrogen administration to thiouracil treated rats might be a result of any one or a combination of the following factors. Since the size of the mammary gland is reduced in rats treated with thiouracil, it might be argued that we are dealing with the effects of a given amount of hormone on a smaller gland. That this is not the case was suggested by Smithcors and Leonard (1942), who reported that the same amount of estrogen administered to a smaller rat with the same size mammary gland as in the case of the hypothyroid rats elicited a normal response, i.e., extension of the duct system.

The other possibilities are that the effectiveness of the injected estrogen is augmented by the hypothyroid state or that the sensitivity of the mammary tissue to injected estrogen is increased by the hypothyroid state. An antagonistic relationship is known to exist between thyroxine and estrogen function (Tyndale and Levin, 1937), and the hyperthyroid state increases the threshold of response to estrogen (Meyer and Wertz, 1938b). Injection of estrogen will lower basal metabolic rate of rats as much as 50 per cent (Sherwood and Bowers, 1936).

It is possible that estrogen in the normal animal may have the potential power to produce both duct and alveolar growth, but the power to produce the latter may be held in check by the functioning of the thyroid. Since the amount of estrogen used in the present work was shown to be capable of stimulating a limited amount of lobule-alveolar growth in the castrate rat, it is possible that the altered metabolic state brought about by thiouracil administration accentuated this response of the mammary gland to injected estrogen. While the present experiments were not designed to discriminate between these two possibilities, it appears likely that both of these factors are operative. If one of the effects of thyroxine in the normal animal is to hold in check the inherent ability of estrogen to produce lobule-alveolar growth, its absence in the hypothyroid state would favor lobule-alveolar development in a mammary gland known to be capable of making this response to injected estrogen.

The results of adrenalectomy on the mammary growth of castrate rats differ somewhat from those obtained by several other workers. The thin atrophic ducts found in the present work resemble those reported by Trentin and Turner (1947), except that in the present work the ducts are very much longer. Reeder and Leonard (1944), reported an increase in the number of lateral buds and found some increase in end bud growth in their adrenalectomized castrate rats. That end bud growth occurred in the present work is obvious from the length of the ducts. The fact that end buds were not abundant at autopsy indicates that the rate of duct extension had decreased some time prior to autopsy.

The difference in appearance of the mammary glands in the pres-

ent work might be explained by the longer experimental period. Trentin and Turner (1947), used adult male rats which had been castrated for a much longer period of time (41-103 days), and were subjected to adrenalectomy only ten days prior to autopsy. It is apparent that only a small mammary gland was present in these rats at the time of adrenalectomy, and in older rats especially, one would not expect much duct extension in the ten day period unless growth stimulating substances were used. Reeder and Leonard (1944), used young male rats which were autopsied only 10-12 days following both castration and adrenalectomy.

The mammary gland of the young animal has been shown by Reece and Leonard (1941), and Smithcoors and Leonard (1943), to be much more responsive to minimal growth stimuli than that of older animals. In the absence of specific growth stimuli, the lateral buds observed by Reeder and Leonard (1944) in their castrate adrenalectomized rats might have been due to the inherent growth potential of the young animal. Since only slight end bud growth was found in these rats it is apparent that the short experimental period did not permit fullest extension of the duct system.

In the present work with adrenalectomized castrate rats injected with estrogen the mammary glands showed an extensive duct system with very marked lobule-alveolar development. This is not exactly in conformity with previous workers. Reeder and Leonard (1944) found that with injection of estrogen in rats treated as mentioned above, they were able to demonstrate dilatation of stimulated lateral buds and an increase in their number. Trentin and Turner (1947) reported that injection of estrogen caused noticeable duct stimulation, but was relatively ineffective in stimulating mammary alveolar development in adrenalectomized castrate rats.

The results of estrogen administration to adrenalectomized castrate rats in the present work confirms substantially those of Reeder and Leonard (1944). In both cases an extensive gland with marked lobule-alveolar development which exceeded that of the estrogen injected castrate rats was found. The relative ineffectiveness of estrogen in stimulating mammary growth in adrenalectomized castrate rats reported by Trentin and Turner (1947) may again be due to differences in experimental procedure.

It was mentioned above that the effect of the thyroid in the normal animal may be to favor duct extension and inhibit lobule-alveolar development. Conversely, the effect of the adrenals in the normal animal may be to restrict duct extension and favor lobule-alveolar development since adrenalectomy causes an exaggeration of the condition found in the young animal with thyroids intact.

The mammary structure of adrenalectomized, thiouracil treated castrate rats has not previously been described. Certain of the effects of both procedures were observed in these rats in the present work.

The duct system of the mammary gland was short as in the case of the thiouracil treated castrate rat and thin as in the case of the adrenalectomized castrate rat. Apparently thiouracil inhibited the extension of the duct system observed after adrenalectomy, and conversely, adrenalectomy inhibited the thickening of the duct system caused by thiouracil treatment.

When estrogen was administered to thiouracil treated, adrenalectomized castrate rats the degree of duct extension was similar to that of the thiouracil treated castrate rats which received estrogen, but the lobule-alveolar development was greater than in the latter group. The greater degree of lobule-alveolar development of this group is compatible with the findings that estrogen administered to thiouracil treated and to adrenalectomized castrate rats stimulated lobule-alveolar development. While these glands resembled those of the thiouracil treated castrate rats which received estrogen, the additional effect of adrenalectomy could be distinguished by the greater distention of the peripheral alveoli.

Since the effects of thiouracil treatment and of adrenalectomy on mammary growth of castrate rats are diametrically opposed, it is obvious that when these techniques are combined the resulting mammary growth represents a compromise between the several forces acting upon the mammary gland. It is well known that any factor which inhibits growth in general has its greatest effect on cells which are most actively growing at the time this inhibition is applied. The most active cells in the mammary glands of the young animal are those at the ends of ducts, thus thiouracil which inhibits growth in general would most likely affect end bud growth. The residual growth potential of the mammary gland would then be seen in a thickening of the ducts which were already present. The administration of a potent growth stimulating substance such as estrogen, would also affect structures already present instead of causing new growth. This explanation would seem to account for the thickening of the shortened ducts found upon thiouracil administration, and the lobule-alveolar development of this less extensive gland upon estrogen administration.

The fact that duct extension is the characteristic response of the mammary gland after adrenalectomy, indicates that some inhibiting factor upon growth of the mammary gland has been removed; thus the more rapidly growing part of the gland, i.e. the duct end buds, are stimulated more than the duct system which has already passed its peak of growth. Since in the present experiments the ducts had apparently reached their definitive size at the time estrogen was administered, it is logical that the greatest effect of estrogen would be to stimulate lobule-alveolar development.

In the adrenalectomized castrate rats which received thiouracil, it is apparent in both body growth and in growth of the duct system

of the mammary gland that the thiouracil was highly effective in producing a hypothyroid state. In the absence of duct extension it is obvious that estrogen administration to these animals was most effective in producing lobule-alveolar development. A partial effect of adrenalectomy in counteracting the inhibition of end bud growth was seen in the greater peripheral distention of the alveoli.

SUMMARY

Thiouracil fed to castrate rats at the rate of 0.1 per cent of ration for 45 days resulted in a shortened and thickened mammary duct system. The administration of estrogen during the last ten days of the thiouracil treatment period resulted in a mammary gland showing shortened and thickened ducts and considerable lobule-alveolar development.

The mammary glands of rats which had been adrenalectomized for 55 days had long atrophic ducts with no lobule-alveolar development. When estrogen was administered for the last ten days of the experimental period, the mammary glands were very extensive in area and showed lobule-alveolar development in excess of that of the estrogen treated castrate rats.

The mammary glands of adrenalectomized, thiouracil treated castrate rats showed a very short atrophic duct system. The administration of estrogen resulted in a mammary gland with a short thick duct system and considerably more lobule-alveolar development than any other group.

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A FURTHER STUDY OF THE ESSENTIALITY OF THE ADRENAL CORTEX IN MEDIATING THE METABOLIC EFFECTS OF ADRENO- CORTICOTROPHIC HORMONE

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FOLLOWING the isolation of adrenocorticotrophic hormone (ACTH) in homogeneous form the results of studies on adrenalectomized animals have consistently indicated that ACTH is a true target hormone for which the adrenal cortex mediates all metabolic effects. Proof that ACTH acts only upon its target organ is not complete. Evans (1947) has noted that the operation of target organ hormones otherwise than through their target organ is becoming increasingly well recognized. Dougherty and White (1944) found that ACTH caused an increase in polymorphonuclear leucocytes in the adrenalectomized rat but this was thought to represent a non-specific response. Dr. Fuller Albright (1947) has suggested that although ACTH may fail to act in the untreated adrenalectomized animal it might synergize the action of adrenal cortical hormones and cause metabolic changes in adrenalectomized animals treated with the cortical hormones. We have reported other examples (Ingle, 1943; Ingle, Ward and Kuizenga, 1947) of responses which were thought to be mediated by an increase in the secretory activity of the adrenal cortices and which were found to occur in the adrenalectomized animal treated with adrenal cortex extracts.

In the present study ACTH was administered to partially depancreatized rats before adrenalectomy and again following adrenalectomy during treatment with adrenal cortex extract. ACTH caused an increase in the excretion of urinary nitrogen and glucose prior to adrenalectomy but not in the adrenalectomized cortical extract-treated rats.

METHODS

Male rats of the Sprague-Dawley strain which were completely free from infections were used. The stock diet was Friskies Dog Cubes. At a weight of

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approximately 300 grams the animals were partially depancreatized by the method of Ingle and Griffith (1942). Following recovery from operation the animals were placed in the metabolism cages and fed a medium carbohydrate diet made according to Table I. All of the animals were force-fed by

TABLE I. MEDIUM CARBOHYDRATE DIET

Constituent	Grams
Cellu flour (Chicago Dietetic Supply)	120
Osborne & Mendel salt mixture	40
Diet yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Vitamin K (2-methyl-1,4-naphthoquinone)	100 mg.
Mazola oil	200
Casein (Labco)	160
Starch	200
Dextrin	190
Sucrose	200
Water to make total of	2000 cc.

stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The techniques and diet were modifications of those described by Reinecke, Ball and Samuels (1939). During the period of adaptation to forced feeding, the amount of diet was increased gradually to prevent the development of "food-shock." The animals were brought to a full feeding of 26 cc. of diet per day on the seventh day. The experiments were carried out in an air-conditioned room with temperature at 74 to 78 degrees F. and humidity at 30 to 35 per cent of saturation. Twenty-four hour samples of urine were collected at the same hour (8:00 to 8:30 A.M.) and were preserved with thymol and citric acid (1 gram per sample) to insure the acidity of the urine for nitrogen analyses. Urine glucose was determined by the method of Benedict (1911) and the determination of urinary non-protein nitrogen was by the micro-Kjeldahl procedure as follows: Proteins were precipitated as the salts of tungstic acid by the Folin-Wu procedure; the ammonia was distilled off into a standard acid solution and titrated with standard base.

The ACTH was prepared by procedure of Li, Evans and Sinipson (1943). It was administered by subcutaneous injection. In Experiment 1, ACTH was given in daily amounts of 0.25 mg. in 3 divided injections for 3 days, 0.5 mg. in 3 divided injections for 3 days and 1.0 mg. in 7 divided injections for 7 days. In Experiment 2, ACTH was given in the daily amount of 1.0 mg. in 7 divided injections for 7 days. In Experiment 3, ACTH was given in the daily amount of 2.0 mg. in 7 divided injections for 7 days.

During the experiment each animal was adrenalectomized by the method of Ingle and Griffith (1942). Sterile technique was used and infections were successfully avoided in all but one animal which was discarded because of abscesses. Following adrenalectomy each animal was treated with beef adrenal extract (Upjohn) in amounts of 3 cc. per day given in 2 divided doses by subcutaneous injection. This extract represented 40 grams of gland per cc. and was free from alcohol. Following adrenalectomy the daily doses of 1.0 and 2.0 mg. of ACTH were repeated in 7 divided injections per day for 7 days.

EXPERIMENTS AND RESULTS

In Experiment 1 (Fig. 1) 4 partially depancreatized rats without spontaneous glycosuria were given ACTH in daily amounts of 0.25 mg. for 3 days, 0.5 mg. for 3 days and 1.0 mg. for 7 days. A rise in

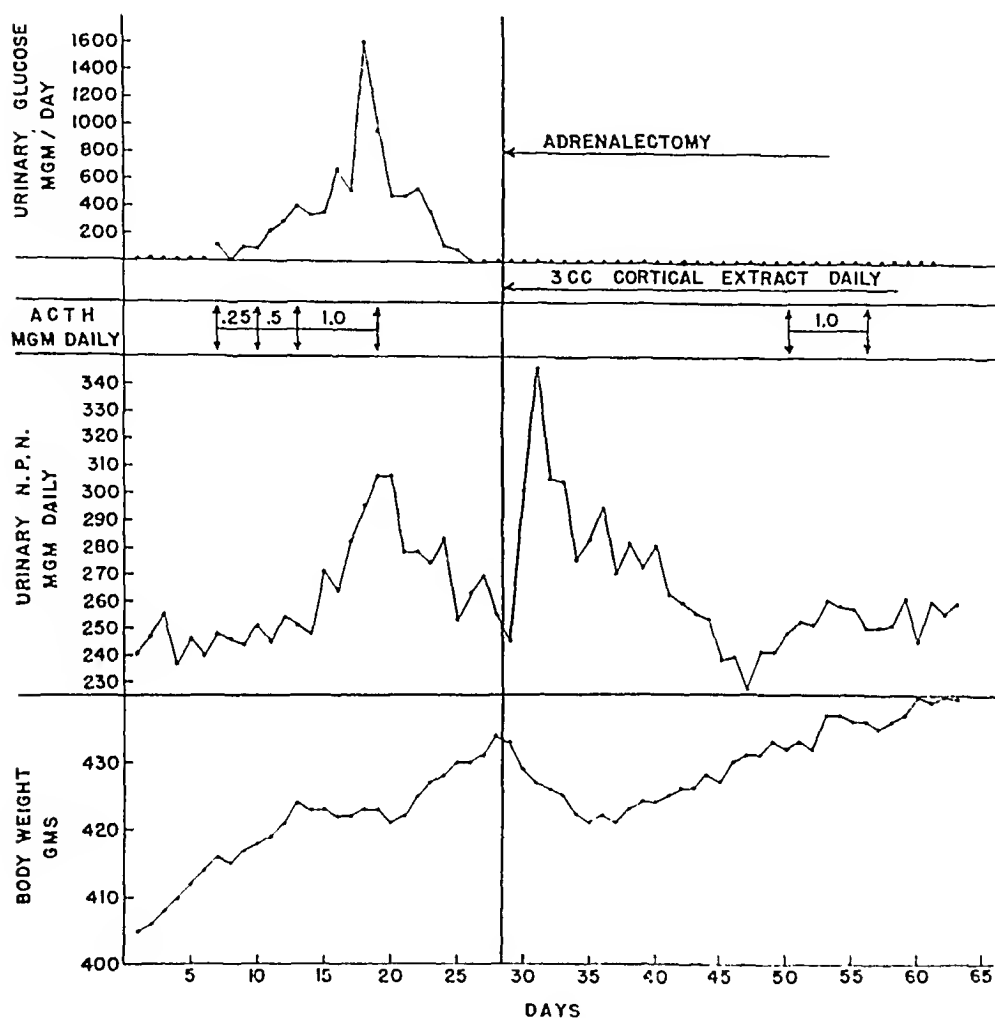


FIG. 1. Averages for 4 partially depancreatized rats treated with ACTH before and after adrenalectomy.

urinary nitrogen and a mild glycosuria was elicited in each rat. When the injections of ACTH were stopped the glycosuria disappeared and the level of urinary nitrogen fell to normal values. The animals were adrenalectomized and maintained with adrenal cortex extract for the remainder of the experiment. Following operation there was a marked rise in nitrogen loss in each animal. The peak was reached on the third postoperative day and gradually fell until the nitrogen balance became strongly positive. Twenty-one days following adrenalectomy each rat was again treated with 1 mg. of ACTH per day for 7 days. None of the rats excreted glucose in response to ACTH and the values

for nitrogen loss did not change to any significant extent. In Experiment 2 (Fig. 2) 2 partially depancreatized rats having mild spontaneous glycosuria showed a marked rise in both urinary glucose and nitrogen in response to 1 mg. of ACTH per day for 7 days. During the second control period the glycosuria disappeared and the amounts of urinary nitrogen decreased to normal values. Following

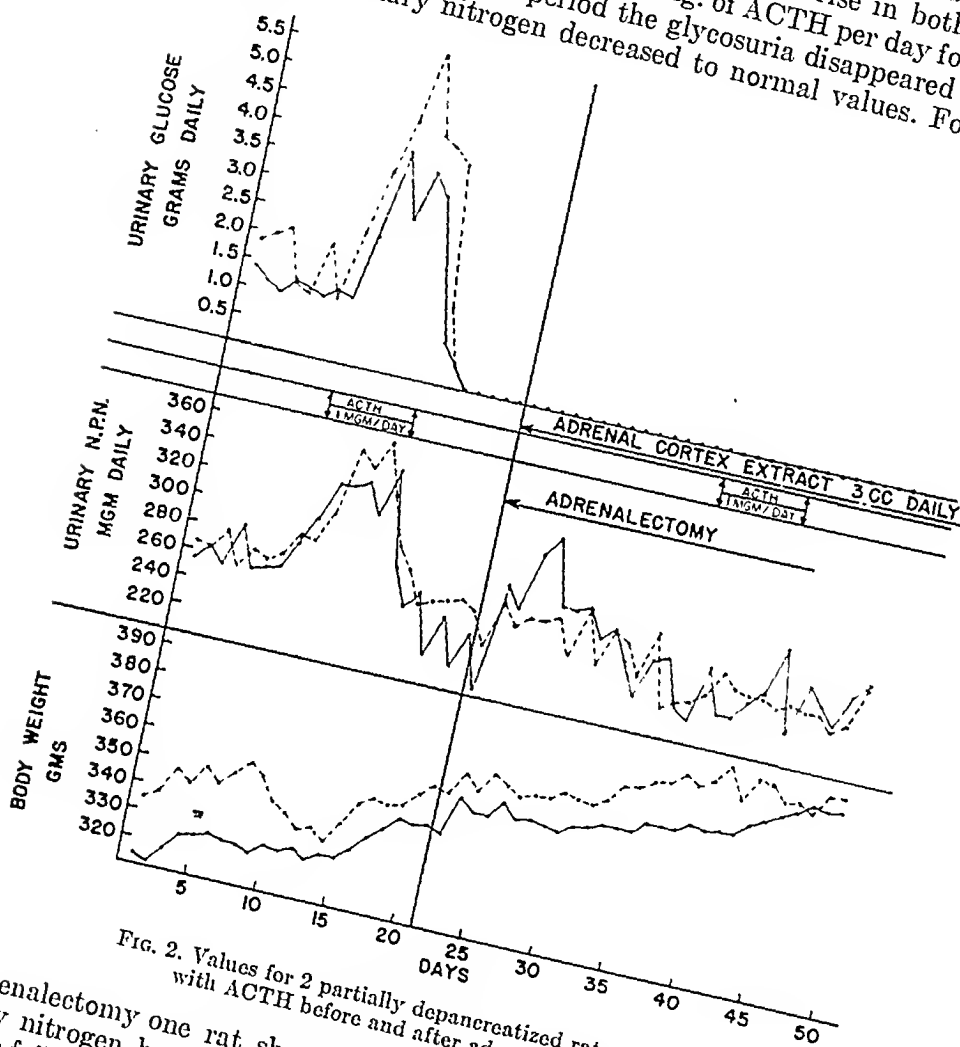


Fig. 2. Values for 2 partially depancreatized rats treated with ACTH before and after adrenalectomy.

adrenalectomy one rat showed a marked temporary increase in urinary nitrogen but the second rat showed little change. Twenty-one days following adrenalectomy each rat was again treated with 1 mg. of ACTH per day for 7 days without causing a glycosuria or an increase in nitrogen loss.

In Experiment 3 (Fig. 3) 1 partially depancreatized rat which did not excrete glucose during the control period developed a marked glycosuria and a rise in urinary nitrogen as the result of giving 2 mg.

day for 7 days. When the injections were stopped the glycosuria and a positive nitrogen balance was re-established. There was a temporary increase in nitrogen loss following ad-

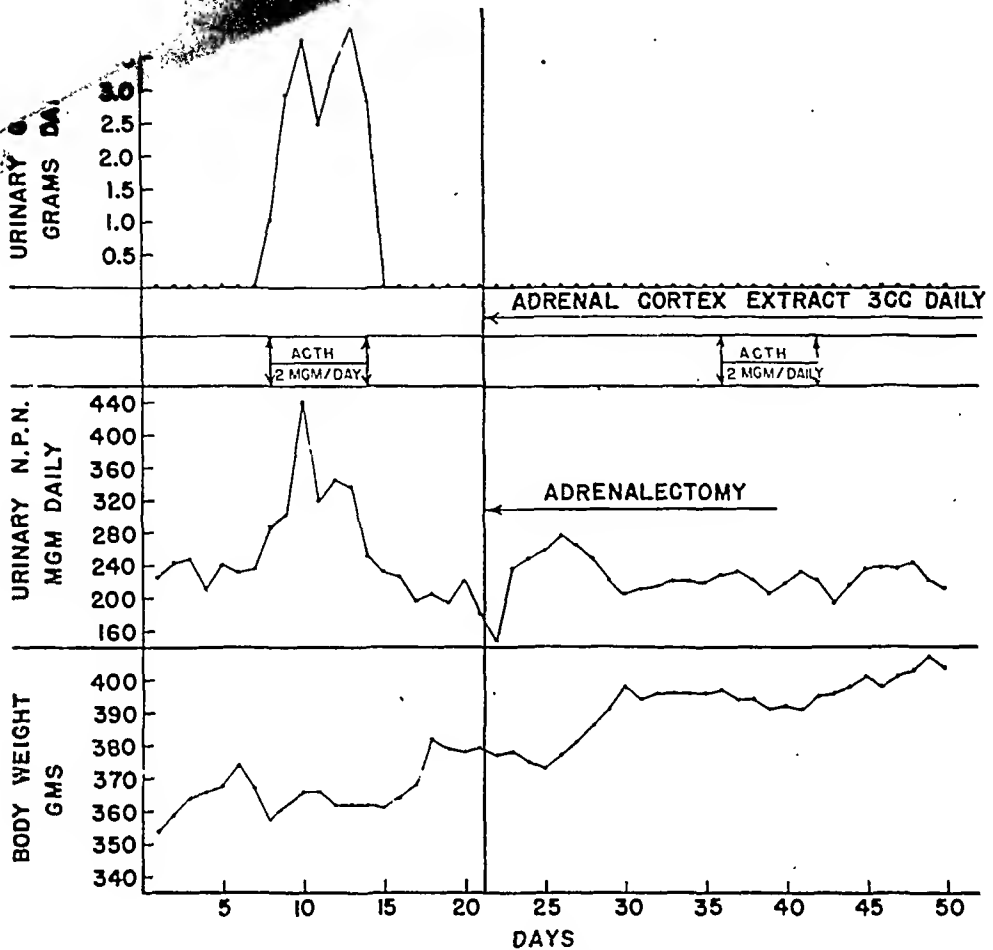


FIG. 3. Values for 1 partially depancreatized rat treated with ACTH before and after adrenalectomy.

renalectomy. Twenty-one days following operation the animal was treated with 2 mg. of ACTH per day for 7 days without causing glycosuria or any significant change in urinary nitrogen.

DISCUSSION

These data support the conclusion that the diabetogenic effect of ACTH and its effect upon nitrogen balance is mediated by the adrenal cortices and that there is no synergism in action.

This is the first demonstration of a diabetogenic effect of pure ACTH in the partially depancreatized rat but it has been fully anticipated by the demonstration that ACTH exacerbates alloxan

diabetes in the rat (Bennett and Li, 1947) and causes glycosuria in the intact, force-fed rat (Ingle, Li and Evans, 1946).

In earlier studies (Ingle and Oberle, 1946; Ingle, Ward and Kuizenga, 1947) we have observed that adrenalectomy in the force-fed rat is followed by a temporary negative nitrogen balance. This was especially marked in the 4 animals of Experiment 1 (Fig. 1) possibly because the operation of these heavier animals caused more trauma to the tissues. It is of interest that this period of increased protein catabolism was not accompanied by glycosuria in any case.

SUMMARY

This study was carried out to test the hypothesis that adrenocorticotrophic hormone may exert an effect upon organic metabolism in the absence of the adrenal cortices by acting synergistically with exogenous adrenal cortical hormones. Partially depancreatized rats which were force-fed a medium carbohydrate diet responded to the administration of ACTH by an increased loss of urinary glucose and non-protein nitrogen when tested prior to adrenalectomy. Following adrenalectomy the animals were maintained on 3 cc. of beef adrenal extract per day during the remainder of the experiment. Under these conditions the administration of ACTH caused no glycosuria or change in the level of urinary nitrogen.

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ALTERATION OF THE ESTRUAL CYCLE IN SHEEP BY USE OF PROGESTERONE AND ITS EFFECT UPON SUBSEQUENT OVULATION AND FERTILITY¹

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CORPORA LUTEA induced in the ovaries of sheep during the breeding season by use of pituitary gonadotropic extracts did not alter the rhythm of the estrual cycles in a uniform manner. Their next heat periods did not all occur at the same time irrespective of the stage in the estrual cycle at which the corpora lutea were produced (Casida, Dutt and Meyer, 1945). Thus, it would appear that the mechanisms for maintenance of the corpora lutea tend to be synchronized with the corpora lutea of the last heat rather than the artificially produced ones.

The effect of corpora lutea on the cyclic rhythm has been demonstrated in several species. Williams and Williams (1921), Hammond (1927) and McKenzie and Terrill (1937) reported that removal of the functional corpora lutea from the ovaries of the cow and the ewe will hasten the time of onset of estrus. Also, Selye, Browne and Collip (1936) and Phillips (1937) reported that daily subcutaneous injections of sufficient amounts of crystalline progesterone into normal female rats resulted in cessation of cyclic rhythm, which was resumed within 2 to 4 days after the end of injections. Injection of crystalline progesterone has been reported to inhibit ovulation induced by electrical stimulation of the cervix in rabbits (Makepeace, Weinstein and Friedman, 1937) and in the rat (Astwood and Fevold, 1939). These workers believe that the effect of progesterone is directly on the pituitary gland in inhibiting the production or the release of the luteinizing hormone, since when this hormone was injected into progesterone-treated rats and rabbits, ovulation did take place.

The work reported here is an attempt to inhibit ovulation in a flock of breeding ewes by injections of crystalline progesterone for a period of time until the ovaries of all animals are free of luteal tissue. Upon cessation of the injections of the inhibiting agent, then all ewes might show estrus at approximately the same time.

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REPRODUCTION IN SHEEP
PLAN OF THE EXPERIMENT

A farm flock of high grade Shropshire ewes was purchased at the beginning of the 1947 breeding season. Each animal was permitted to go through at least one complete estrual cycle before treatment was started. Control data for each ewe were thus furnished by the length of the estrual cycle and estrus was determined by checking twice daily at twelve-hour intervals with an aproned ram, and a ewe was considered to be in heat only if she allowed the ram to mount and remained standing after he had mounted. Checking for the occurrence of estrus was continued in this manner throughout the treatment period and also the post-treatment period until time of autopsy.

An experiment was designed using 30 breeding ewes on two different levels of injection; these were started at three stages of the cycle, i.e., four, eight and 12 days after the first day of estrus. Eighteen ewes, six in each stage were given daily injections of 5 mg. of progesterone and 12 ewes, four in each stage were given 10 mg. daily. Treatment consisted of 14 consecutive daily subcutaneous injections in all ewes. It was necessary to continue injections for 14 days, since the functional life of luteal tissue in the ewe is approximately of this duration. And, in order to align all ewes so that their ovaries would be free of functional luteal tissue, treatment would have to continue for at least that length of time.

All ewes on the experiment received daily injections of 5 c.c. of pure corn oil with the respective amounts of crystalline progesterone² dissolved in the oil. Thus, the ewes on the 5-mg. dosage received a total of 70 mg. of progesterone, while those on the 10-mg. dosage received a total of 140 mg.

An observational laparotomy was performed on each ewe on the day following the last injection to determine whether ovulation had occurred under treatment even though estrus was suppressed. At the same time the numbers of corpora lutea resulting from the last natural estrus and the sizes of the largest follicles present were also recorded. The positions of the largest follicles in the ovaries were marked at the time of laparotomy by inserting a needle dipped in India ink just beneath the ovarian tunic at several points at the base of the follicle. Precaution was observed to avoid rupturing a follicle. On autopsy it was then possible to tell definitely whether such follicles had ovulated. All ewes showing estrus after the end of treatment were artificially inseminated with good-quality, undiluted semen. These animals were slaughtered 36 to 48 hours after the end of estrus, and the reproductive tracts were immediately taken to the laboratory where a thorough search was made for ova. Those ewes which did not show an estrus after treatment were slaughtered seven days after the end of treatment, regardless of whether they had ovulated during the period of treatment.

RESULTS AND DISCUSSION

None of the 30 ewes on the two dosages showed estrus during the period of injection. On observational laparotomy the day after the end of treatment, however, it was found that three of the ewes on the

² The authors are indebted to the Glidden Company, Chicago, Illinois, for the generous gift of crystalline progesterone for this work.

5-mg. dosage had recently ovulated, even though they had exhibited no estrus. Ovulation was inhibited in the remaining 15 ewes on this dosage and in all of the ewes on the 10-mg. dosage.

The corpora lutea from the last natural estrus had regressed in size to a diameter of 1.5 to 3 mm. in all ewes except one on the 5-mg. dosage, in which a 5 mm. corpus was observed. This ewe did not show a post-treatment estrus; however, at the time of autopsy she had a recent corpus luteum indicating a "quiet" ovulation.

Influence of Stage on Effectiveness of Treatment

Estrus and ovulation both occurred in only 12 of the 18 ewes on the 5-mg. dosage within seven days subsequent to the termination of treatment. Three of the remaining six ewes ovulated during treatment and consequently showed no estrus during the post-treatment period. Of the remaining three ewes, two were considered to have cystic follicles at the time of autopsy. One of these had a 22-mm. follicle on the left ovary; however, this ewe did show estrus for a period of one day beginning three days after the last injection. The other ewe had a 23-mm. follicle on the right ovary, but she did not show any signs of estrus as indicated by the action of the teaser ram during the post-treatment period. The one remaining ewe on this dosage showed no post-treatment estrus; however, on autopsy it was found that a quiet ovulation had occurred.

The stage at which treatment was initiated appears to have an effect on the inhibition of ovulation during treatment with this dosage. Ovulation was inhibited in all ewes in which treatment was begun at the 4-day stage, in 5 out of 6 in which treatment was begun at the 8-day stage and in 4 out of 6 at the 12-day stage. With the ewes started at the 4-day stage the animal's own luteal tissue has to be supplemented for only 3 or 4 days, while in the 8- and the 12-day stages, suppression of the pituitary luteinizing hormone is dependent for a longer period of time upon only the exogenous progesterone.

Effect upon Interval from End of Treatment to Estrus

The regularity of the occurrence of post-treatment estrus in the ewes on the 10-mg. dosage is very striking. The average interval from the end of treatment until the onset of next estrus in this group was 3.2 days with a range of 0.5 day, while the average predicted from the control intervals was 11.8 days (Table 1).

The "typical" ewes on the 5-mg. dosage, those showing a post-treatment estrus and ovulation, had an average interval to onset of estrus of 2.8 days with a range of 2.0 days. Considering all of the ewes on this dosage, and, assuming that those which did not show estrus would have done so at the next predicted estrual period, the average interval to estrus for the 18 ewes would have been 5.4 days. The average predicted interval on this dosage was 12.1 days. Two of the ewes

had cystic follicles in their ovaries, and it is not known what effect, if any, this condition may have had on the time of onset of the next estrual period. Grant (1934) reported that cystic follicles are a rare occurrence in the ewe, and when they are present, they do not have any effect on the estrual cycle. Phillips (1937) has reported that a threshold dosage of progesterone in the rat resulted in a delay in return to normal cyclic rhythm after treatment; contrasted with this, the return to normal cyclic

TABLE 1. EFFECT ON LENGTH AND UNIFORMITY OF INTERVAL FROM END OF TREATMENT UNTIL NEXT ESTRUS.

Interval and Dosage	Stage of Treatment							
	4-day		8-day		12-day		All	
	Mean	Variance	Mean	Variance	Mean	Variance	Mean	Variance
5-mg.* Predicted	15.9	3.8	12.2	1.4				
Observed	4.7	25.7	5.8	21.7	8.3	0.9	12.1	11.9
5-mg.† Predicted	16.1	3.6	12.4	1.7	5.8	9.3	5.4	17.0
Observed	2.5	0.1	2.9	0.7	8.2	0.3	12.9	13.1
10-mg. Predicted	16.1	1.4	11.2	0.9	3.2	0.1	2.8	0.3
Observed	3.1	0.1	3.2	0.1	8.0	0.5	11.8	12.9
					3.1	0.1	3.2	0.1

* Includes all ewes on the 5-mg. dosage.

† Includes only the 12 ewes showing post-treatment estrus and ovulation.

rhythm occurred within 3 to 5 days when higher dosages were used. Although Phillips did not determine whether cystic follicles had formed or whether the rats had ovulated without showing estrus, it may be that the threshold dosage in the rats had resulted in the formation of cystic follicles, which would explain the delay in return to normal rhythm. The occurrence of cystic follicles in two of the ewes and their failure to show a post-treatment estrus may be characteristic of a threshold dosage of progesterone.

The average interval to the beginning of estrus for the typical ewes in the 5-mg. group was 2.8 days and differed from the 10-mg. group for which the average interval was 3.2 days. A longer interval to onset of estrus might be the result of the higher dosage of progesterone more completely suppressing the output of luteinizing hormone from the pituitary gland.

In Table 2 the average lengths of the treatment estrual cycles of the ewes showing a post-treatment estrus are given. For the ewes started on treatment at the 12-day stage on the 5-mg. dosage, the average experimental cycle length was 27.7 days and for those on the 10-mg. dosage, 27.9 days. The average length of the experimental cycles was slightly longer for the ewes on the 10-mg. dosage due to a longer interval from the end of treatment until the onset of estrus.

TABLE 2. EFFECT ON LENGTH OF ESTRUAL CYCLE

Dosage	Cycle	Stage of Treatment					
		4-day		8-day		12-day	
		Mean	Variance	Mean	Variance	Mean	Variance
5-mg.	Control	16.4	1.1	16.5	0.5	16.5	0
	Experimental	19.4	0.2	23.5	0.5	27.7	0.1
10-mg.	Control	16.5	0.5	16.1	0.2	16.4	0.1
	Experimental	20.0	0.2	24.2	0.1	27.9	0.1

The experimental cycles were longer than the control cycles for all of the ewes, regardless of the stage at which treatment was begun. The experimental period of the animals was terminated shortly after the occurrence of a post-treatment estrus; therefore, no information was obtained on the subsequent cyclic rhythm.

Duration of Estrus

The length of the post-treatment estrus was shorter than the control by an average of 0.2 day for the ewes on the 5-mg. dosage and by 0.1 day for the ewes on the 10-mg. dosage. Neither difference was statistically significant. Physiologically a shorter estrual period might be expected if the suppression of the luteinizing hormone had not been complete, since under the augmentative influence of a small amount of luteinizer the follicles would be more mature at the end of treatment than if no luteinizer were present. It was pointed out earlier that the interval to onset of estrus was significantly shorter for the ewes on the 5-mg. dosage and, although there is no significant difference in the duration of estrus, the ewes on the lower dosage did have the shorter estrual period.

Further evidence for supporting the hypothesis of a "blockage" of the luteinizing hormone by progesterone is furnished by the differences in follicular size observed at laparotomy in the two groups of ewes. The average diameter of the largest follicle in the ovaries of the typical ewes on the 5-mg. dosage was 9.0 mm. and for the ewes on the 10-mg. dosage, 6.8 mm. (Table 3). This difference between dosages

TABLE 3. EFFECT OF TWO DIFFERENT DOSAGES OF PROGESTERONE ON SIZE (mm.) OF LARGEST FOLLICLE AT END OF TREATMENT

Dosage	Stage of Treatment							
	4-day		8-day		12-day		All	
	Mean	Variance	Mean	Variance	Mean	Variance	Mean	Variance
5-mg.	8.3	0.3	9.8	1.0	8.8	4.9	9.0	1.8
10-mg.	6.5	1.0	7.0	0.7	7.0	2.0	6.8	1.1

was highly significant; however, the stage at which treatment was started did not show any significant effect on follicular size when observed at the time of laparotomy. The large follicles in the ewes on the 5-mg. dosage and also the shorter interval from end of treatment to ovulation (assuming ovulation occurs at or near the end of estrus) can be interpreted as a less effectual blockage of the luteinizer by this dosage. The amount of luteinizer was high enough to precipitate ovulation in three of the ewes on this dosage during the treatment period.

Possible explanations for the cause of the cystic follicles observed in two of the ewes on the 5-mg. dosage at time of autopsy are: (1) The amount of luteinizer being secreted by the pituitary gland during the treatment period was high enough to augment the action of the

TABLE 4. PERCENTAGE OF THE LARGEST FOLLICLES OBSERVED AT LAPAROTOMY WHICH LATER OVULATED

Dosage	Stage of Treatment			
	4-day	8-day	12-day	All
5-mg.	83	100	37	75
10-mg.	25	50	25	33

follicle-stimulating hormone, causing the follicles to assume mature size without ovulating—as a result of this augmentation. they may no longer have been able to react to the luteinizer with the ovulation response when the progesterone was withheld and continued to grow and became cystic; (2) The continual loss of luteinizer had so depleted the pituitary gland that after progesterone injections were stopped, the pituitary was unable to raise the level of luteinizer high enough to cause ovulation.

In order to throw more light on the nature of the ovarian responses the largest follicle observed in each animal at laparotomy was marked so that its status at the time of autopsy could be ascertained. The largest follicle so marked ovulated in 75 per cent of the ewes on the 5-mg. dosage (Table 4), and in only 33 per cent of the ewes on the 10-mg. dosage. In some of the ewes the largest follicle had regressed at time of autopsy so that its location was identifiable only by the India ink marks.

Since the largest follicles were observed in the ewes on the low dosage of progesterone and the percentage of such follicles ovulating was higher in that group, there is no evidence to indicate that the largest follicles are less reactive to the ovulating stimulus than the smaller follicles. In fact, the reverse appears to be true. The cystic follicle observed in one of the two ewes was definitely not the largest follicle observed at laparotomy since it occurred in the opposite ovary. The cystic follicle noted in the other ewe could not be traced

definitely, since at autopsy the follicle was so large and the ovary so distorted in shape that it was not possible to identify this follicle as the same one that was marked at laparotomy. The suggested cause of the cystic follicles then is the lowness of the level of luteinizer interacting with the follicle-stimulator, resulting in continued follicular enlargement.

Possible explanations for failure of the ewes to show estrus when they ovulated during the injection period are: (1) that while in the blood stream progesterone inactivates estrogen or facilitates its excretion from the body at such a rapid rate that it does not act on the nervous system or (2) that progesterone itself acts on the nervous system, rendering it insensitive to the stimulus of estrogen. An anesthetic action of high dosages of progesterone on the nervous system of the rat has been reported (Selye, 1941; Hartman, Burge and Doctor, 1947).

Effect on Ovulation

One effect of inhibition of ovulation for a period of time might be a change in the number of ovulations at the next estrual period. An increased number of follicles might be ovulated following inhibition of the pituitary output of luteinizer as a result of the ovaries being

TABLE 5. EFFECT ON NUMBER OF OVULATIONS

Dosage	Estrual Period	Stage of Treatment			
		4-day	8-day	12-day	All
5-mg.	Control	2.2	2.0	1.3	1.9
	Experimental	1.6	1.5	1.0	1.4
10-mg.	Control	1.8	1.8	1.8	1.8
	Experimental	1.5	1.8	1.8	1.7

subjected to an extended period of follicle-stimulating action followed by release of an accumulated reserve of luteinizing hormone. As control data, the numbers of corpora lutea present from the last estrual period were taken. In the analysis of the data for the 5-mg. group only the typical ewes will be considered.

The average number of ovulations for the ewes on the 5-mg. dosage was significantly lowered at the experimental estrual period by an average of 0.5 per ewe. For the ewes on the 10-mg. dosage there was no significant change, 1.7 per ewe for the experimental period and 1.8 for the control period (Table 5). The smaller number of ovulations in the 5-mg. group can also be explained by (1) an insufficient amount of luteinizer after withdrawal of progesterone or (2) some of the potentially ovulatable follicles were spoiled by the low level of luteinizer which was being released continually. The former possi-

REPRODUCTION IN SHEEP

bility presupposes that the output of luteinizing hormone by the pituitary gland is limited and that a gradual release will result in depletion. There is little or no evidence, at least from the percentages of the largest follicle ovulating in the ewes on the two dosages, to indicate that any of the follicles had been spoiled. In the 10-mg. dosage the suppression of luteinizer was perhaps more complete during the treatment period so that a normal level resulted when the inhibiting agent was withheld.

Effect on Fertility

It is possible that the eggs ovulated after lengthening the estrual cycle (in the case of some of the ewes to as much as 28 days) may be aged or that their environment may be changed deleteriously so that they are no longer potentially fertile. Since there are no control data on the fertility of these ewes, the main points that can be studied are whether the eggs ovulated after the treatment are fertilizable and whether dosage and stage have any effect upon the fertility of the eggs. Of the eggs recovered (Table 6) from the ewes on the 5-mg.

TABLE 6. FERTILITY OF EGGS

Stage	Dosage					
	5-mg.		10-mg.		Both	
	No. Eggs	% Fert.	No. Eggs	% Fert.	No. Eggs	% Fert.
4-day	8	50			14	64
8-day	5	20	6	33	11	27
12-day	3	67	6	83	9	77
All	16	44	18	67	34	56

dosage, 44 per cent were cleaved (2 to 8 cells) and all appeared to be normal at the time of recovery except one 4-celled egg which appeared to be in the early stages of degeneration. In this group eggs were recovered from 12 ewes, six of which yielded fertile eggs and the remaining six, only unfertilized eggs. On the 10-mg. dosage 67 per cent of the eggs recovered were fertilized (4 to 12 cells) and all appeared to be normal. Eggs were recovered from 11 out of the 12 ewes on this dosage and 10 of them yielded at least one fertile egg. The numbers are too few for statistical analysis, but the lower fertility in the ewes on the 5-mg. dosage again suggests that this dosage had resulted in a greater physiological disturbance.

There was possibly a difference in fertility of the eggs recovered from ewes when treatment was started at the different stages of the cycle. The fertilizability of the eggs recovered from the ewes started at the 8-day stage was lower on both dosages than for the eggs recovered from either the 4-day or the 12-day-stage ewes. It is difficult to visualize any physiological basis for such a difference since the re-

action of the 8-day ewes to the treatment was as uniform in the interval from end of treatment to estrus and in the length of the post-treatment estrus as were the ewes which were started at the other two stages of the cycle. It is possible that the difference may be due to chance. The point of real importance is that the eggs resulting from the first estrual period following progesterone treatment are in general fertile. Whether they would have gone on to develop into normal embryos was not determined.

SUMMARY

The effects of crystalline progesterone on regulating the estrual cycle of the ewe during the active breeding season was studied. Thirty ewes were given daily subcutaneous injections starting on the fourth, eighth and twelfth days after the first day of estrus. Two dosages were used, one of 5 mg. and one of 10 mg. daily. Eighteen ewes were placed on the first dosage and 12 ewes on the second dosage.

Estrus was suppressed during treatment with either dosage. Three of the ewes on the 5-mg. dosage ovulated during treatment, but ovulation was inhibited during treatment in all ewes on the 10-mg. dosage. Estrus occurred in all 12 ewes on the 10-mg. dosage from 3 to 3.5 days after the last injection. The 5-mg. dosage was not so effective in controlling the time of onset of estrus. Only 12 of the 18 ewes showed a post-treatment estrus and ovulation. Two of the ewes had cystic follicles and one a quiet ovulation when they were autopsied.

The length of the post-treatment estrus was shortened, but not significantly with either dosage. In the typical ewes on the 5-mg. dosage the number of post-treatment ovulations was significantly lower than for the control period, average per ewe being 1.4 for the experimental and 1.9 for the control period. The number of ovulations per ewe on the 10-mg. dosage was 1.7 for the experimental and 1.8 for the control period.

As a result of the treatment, the estrual cycles of all the ewes were lengthened. The longest cycles were observed in the ewes on the 10-mg. dosage treated at the 12-day stage where the group average was 27.9 days. The average cycle length for the typical ewes on the 5-mg. dosage at the same stage was 27.7 days. The experimental cycles of the ewes in the other two stages were shorter by approximately the differences between the corresponding stage intervals, i.e., 4 and 8 days.

Fertilized eggs were recovered from ewes treated at all stages of the cycle on both dosages. The higher percentage of fertile eggs, 67 per cent, was recovered from the ewes on the 10-mg. dosage, as compared to 44 per cent from the ewes on the 5-mg. dosage. The eggs recovered from ewes when treatment was started at the 8-day stage showed the lowest fertility.

Daily subcutaneous injections of 10 milligrams of progesterone

were more effective in decreasing the variation in time of occurrence of estrus during the breeding season than were injections of 5 milligrams of progesterone. The time of onset of estrus was controlled in 100 per cent of the ewes on the higher dosage, but in only 67 per cent of the ewes on the lower dosage.

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OBSERVATIONS ON THE SEX SKIN AND SEX CYCLE IN THE CHIMPANZEE¹

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THERE ARE at least four stages involved in the study of the mammalian sex cycle. The first is the establishment of the length of the cycle in the particular species under investigation, the second the subdivision of the cycle into such phases as the periodic fluctuations in morphology permit, the third the study of deviations in the hope of obtaining information that may lead to a better understanding of the normal, and finally the correlation of endocrine changes with the periodic changes in morphology and behavior. In primates the first phase of the investigation is simplified by the almost universal occurrence of menstruation, which provides a reliable initial and terminal indicator. Further, in the chimpanzee and several other species of primates the natural periodic variations in size, color, shape, and the degree of tumescence of the anogenital area afford a second set of indicators which provide a direct perceptual basis for subdividing the sexual cycle. These indicators also make possible the ready recognition of a variety of deviations from the normal.

Previous reports on the sexual cycle of the female chimpanzee (Elder and Yerkes 1936, Young and Yerkes 1943) have contributed greatly to our knowledge of the normal cycle in this organism and have further afforded information on some of the aberrations which may appear. The present paper represents a continuation of the study of the chimpanzee sex cycle with particular emphasis on the nature of spontaneously occurring deviations from the normal. It also reports our attempts to duplicate these divergences from normal periodicity in induced or artificial cycles² in two female castrate chimpanzees. An integration of the data from spontaneously occurring aberrations with the evidence on the artificial induction of atypical cycles seems to provide explanations for deviations from the normal which appear in the first cycles after the menarche, for the occur-

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² The hormones used in this study, Alphaestradiol (Progynon DH), ethinyl estradiol (Estinyl) and anhydrohydroxyprogesterone (Pranone) were generously supplied by the Schering Corp. Bloomfield, N. J.

rence of cyclical changes in the sex skin during pregnancy, and for the mode of reinstitution of cycles after parturition.

THE TYPICAL CHIMPANZEE SEX CYCLE

In contrast to the intensive subdivision of the cycle in the earlier work of Yerkes and Elder (1936), Young and Yerkes (1943) divided the cycle into four phases. We will follow the scheme of subdivision used by the more recent investigators. The full cycle has a mean of 37 ± 0.14 days and the four phases are of unequal duration (Table 1).

TABLE 1. MEAN LENGTHS OF PHASES OF CYCLE AND OF CYCLE IN THE CHIMPANZEE (TAKEN FROM YOUNG AND YERKES, 1943)

Preswelling Phase	Swelling Phase	Postswelling Phase	Menstruation	Entire Cycle
7 ± 0.08	18 ± 0.08	10 ± 0.06	3 ± 0.03	37 ± 0.14

The first or preswelling phase is a period of genital quiescence which begins with the disappearance of the external signs of menstruation. Physiologically the preswelling phase is correlated with either a lack of follicular growth or with a low level of estrogen production.

The second phase or swelling phase of the cycle is believed to coincide with follicular growth. In this phase a swelling of the sex skin involves first the labial and circumanal areas, but gradually tumescence extends anteriorly over the mons pubis and laterally to the callosities. Maximal tumescence is usually attained by the fifteenth day of the cycle. There is much interindividual variation in the appearance of the swelling. In some chimpanzees the enlarged perineum has well-defined boundaries and stands out as a prominent rounded mass, while in other animals the labia are greatly hypertrophied and form the major part of the swelling. The extent to which the mons pubis is involved is variable, and in those animals in which it is most prominent the labia are usually not much enlarged. Sealing and sloughing of the surface epithelium, a feature which heralds regression of the swelling in the baboon (Gillman and Gilbert, 1946), may occur during any portion of the maximal period and in the chimpanzee is not necessarily a harbinger of detumescence. The degree of tension developed shows considerable variation which may be determined by manual pressure but as yet no quantitative measure has been developed. The color of the non-pigmented sex skin ranges through various shades of pink from very pale to rose. Measurements of the swelling circumference, such as Gilman and Gilbert (1946) have found so valuable in the chaema baboon, cannot be made readily in the chimpanzee. Ovulation as determined by impregnation and by direct ovarian examination occurs from one to six days before the onset of detumescence (Elder and Yerkes; 1936, Young and Yerkes, 1943). Thus tumescence continues for a variable period after ovulation and

the onset of detumescence, the third externally visible alteration in the sex cycle, is not concomitant with ovulation. The duration of the lag between the two events ranges from one to six days.

The third phase of the sex cycle is characterized first by detumescence of the ano-genital area and then by a period of genital quiescence which is terminated by menstruation. In ovulatory cycles this phase corresponds to the physiological changes involved in the luteal phase of the cycle. The change from tumescence to detumescence and the entire absorption of the genital swelling occurs in a remarkably short time. The detumescent period may be as short as forty-eight hours with the major decrease in the first twenty-four hours. In the baboon histological studies by Zuckerman (1930) have shown that the swelling is the result of the accumulation of intercellular fluid rather than the product of cellular enlargement. It is probable that the same is true for the chimpanzee because in the few instances where we have observed lacerations of the swelling the tissue has appeared to be quite engorged and there was seepage of clear, colorless fluid from the torn surfaces.

The fourth and final phase of the cycle is menstruation which has an average duration of three days. Thus in the average cycle the preswelling phase occupies seven days, the swelling eighteen days, the postswelling phase ten days, and menstruation three days for a total average (mean) cycle length of about 38 days, with a range of 22 to 187 days. Young and Yerkes (1943) also give the median as 35 days and the mode as 33 days and state that the curve is skewed to the right. Unfortunately they failed to give the median and mode for the various subdivisions of the cycle.

In order better to understand the physiological events in average cycles the study of atypical cycles is necessary. Atypical cycles may be observed under four different sets of circumstances. These are as follows: 1. Variability in the normal cycle (subdivided into a. Introduction, b. Premenstrual swelling, c. No premenstrual quiescence, and d. No preswelling phase), 2. Initial cyclical events at puberty, 3. Changes in sex skin during pregnancy and, 4. Postpartum reinstitution of cycle.

VARIABILITY IN THE NORMAL CYCLE

Introduction. Variations are found not infrequently in the cycles of presumably normal subjects and are listed in Table 2. The first two columns give the name of the animal and the number of her cycles that we have followed. In subsequent columns there are listed: the frequency of premenstrual swelling, of the failure of detumescence to be completed before the onset of menstruation, of the omission of the preswelling phase, and of the occurrence of tumescence during menstruation. These conditions are illustrated in graphic schemata in Figure 1. It is evident that any of the first three conditions may lead

to an engorged sex skin during the catemenia. This occurrence of swelling during menstruation has two aspects. First, detumescence may not be complete or the premenstrual swelling may extend into the period of flow; and second, swelling may begin prior to cessation of

TABLE 2. VARIATION IN THE NORMAL CYCLE

Name	Number of Cycles	Premenstrual Swelling	No Premenstrual Quiescence	No Preswelling Phase	Menstruation and Swelling
Alpha	40	0	0		
Ami	4	0	1		
Bentia	55	11	8	5	0
Bula	31	4	0	0	0
Dina	4	1	0	1	5
Dita	43	3	0	0	0
Fifi	84	5	2	0	0
Gamma	23	0	0	2	0
Helene	33	3	0	10	2
Josie	50	3	4	0	0
Lia	48	1	0	3	0
May	52	9	3	0	1
Mimi	51	2	5	13	1
Pati	73	1	18	3	3
Soda	41	4	5	4	0
Vera	9	0	8	4	6
Wendy	52	5	0	0	1
			5	11	7

flow. Table 2 shows that there is considerable variability among animals with certain individuals contributing the largest number of deviations.

However it should be noted that frequency of variation and utility as a breeding animal have only a slight negative correlation. Alpha, with few deviations and Wendy, with many, have equally good breeding records, but on the other hand Bentia, Dita, Lia, May, Mimi and Pati who have many deviations have had none or very few offspring while every adult animal showing few variations does reproduce satisfactorily.

Premenstrual swelling. When premenstrual swelling (Table 2, Column 3) occurs it is a slight tumescence which seldom attains a size larger than $\frac{1}{2}$ of the usual maximum, begins a few days before the onset of menstrual flow, and usually regresses completely by the second menstrual day (Figure 1a). It is possible that this phenomenon is similar to the condition discovered by Gilbert and Gillman (1944) in the chacma baboon and termed by them the "rebound effect." We have reproduced premenstrual swelling in only one artificial cycle which was originally designed to determine if progesterone withdrawal could produce menstruation in the chimpanzee as in Maccus mulatta (Hisaw and Greep, 1938) when the estrogen dosage was maintained at constant level. Full tumescence was produced by oral administration of alpha estradiol and then anhydrohydroxyprogesterone (pregnen-

inoline) was also given. Three days after complete detumescence the pregneninoline was discontinued while the alpha estradiol was continued with no change in daily dosage. On the third day after the last dose of the progestational hormone the swelling had increased to $\frac{1}{2}$

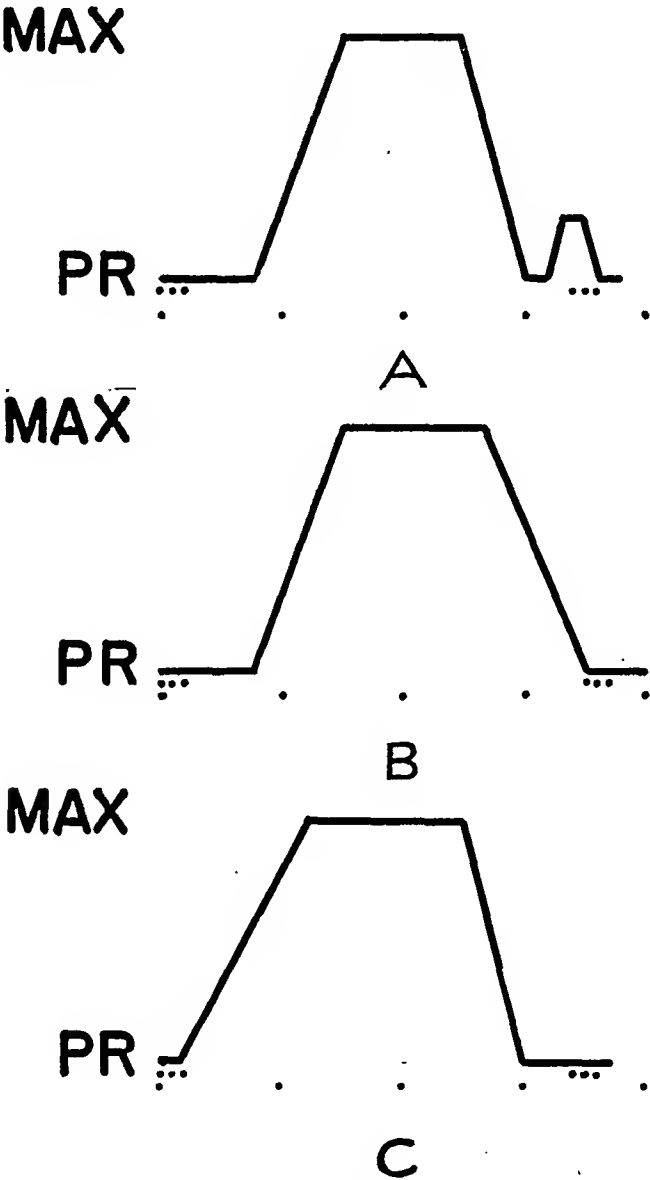


FIG. 1. Schemata illustrating three types of unusual cycles. In these and also in Figures 2, 3 and 4, time is indicated by the horizontal row of dots beneath the graphs. Each of these dots represents an interval of ten days. PR represents the lowest level of sex skin tumescence in the cyclical female and is definitely larger than in the castrate. It is ordinarily seen just before, just after and during menstruation. MAX represents the height of tumescence, the time when the swelling of the sex skin is at a maximum. Menstruation is indicated by the dots immediately beneath the graphs. Figure 1A. Premenstrual swelling. Figure 1B. No premenstrual quiescence. Figure 1C. No preswelling phase.

of the maximum and the following day when menstruation had begun the swelling was $\frac{1}{4}$.

No premenstrual quiescence. In the average cycle detumescence is complete and the sex skin quiescent for at least twenty-four hours prior to the onset of menstruation. This condition was designated simply as the premenstrual phase (Yerkes and Elder, 1936). However, on occasion menstruation begins before the swelling of the sex skin has reached the resting stage (Figure 1b). It may be only a coincidence that Mimi, whose sex skin failed to regress to the resting stage in 35% of her cycles (Table 2, Column 4), was selected for castration because of her worthlessness as a breeder and that Bentia, with no period of postswelling quiescence in 15% of her cycles, also has a very poor breeding record, for Soda, with 20% of such cycles, has a satisfactory record (Nissen and Yerkes, 1943). In no case have we seen tumescence extend beyond the period of flow but it is not infrequent for deturgescence to be incomplete on the last day of bleeding. The other phases of such cycles are usually within the normal range of variation in the same animal. It may be that such cycles are due to partial failure of progesterone production. Artificial cycles in which the progesterone hormone is given at about one half the threshold dose and both the estrogen and the progesten are terminated at the same time yield similar changes in the sex skin. That is deturgescence is not complete before the onset of flow.

No preswelling phase. In the usual cycle the sex skin remains at the quiescent level both during menstruation and for a period of at least one day after cessation of flow. This brief postmenstrual quiescence, which is followed by the onset of tumescence, was designated the postmenstrual phase by Yerkes and Elder (1936) but we prefer preswelling, as it is a more descriptive term. In some cycles it is either markedly shortened or even absent (Figure 1c). A skipping of the preswelling phase (Table 2, Column 5) may mean either that swelling of the sex skin began before the cessation of flow or that there was a noticeable amount of swelling already present on the following day. These conditions have occurred in some of our best breeding animals and apparently bear no relation to fertility. For example Lia was an absolute failure as a breeder and had such cycles 27% of the time while Wendy, with an excellent breeding record, has had 21% of such cycles. Much of interest is revealed by an intensive examination of a series of Wendy's cycles. In Wendy the onset of swelling has often (six times—11% of her cycles) occurred before the end of menstruation. The component phases of these six cycles are given in Table 3. The length of the menstrual period of the preceding cycle is followed by the number of days for which swelling was recorded during flow. Then the subsequent cycle is tabulated in terms of the number of days from the end of menstruation to the beginning of detumescence, the length of the postswelling phase (in the non-pregnant cycles),

the duration of menstruation, and finally the total length of the cycle. Thus in the first cycle listed menstrual flow lasted for three days on the last day of which the sexual skin was definitely in early swelling. The tumescent period extended to the 13th day after cessation of flow, corresponding to a total length of the swelling phase of 14 days. The post swelling phase lasted 13 days and menstrual flow for 2 days with a total length for the cycle of 28 days. In three of these cycles Wendy

TABLE 3. DEVIANT CYCLES OF WENDY AS COMPARED TO HER AVERAGE CYCLE AND HER SHORTER CYCLES*

Menstruation	Old Cycle Menstrual Swelling	Swelling Phase	New Cycle Postswelling Phase	Menstruation	Total
3	1	13	13	2	28
4	2	14	Pregnant		
2	1	14	13	3	30
3	2	13	14	2	27
2	2	13	Pregnant		
3	1	13	Pregnant		
	Preswelling Phase	Swelling Phase	Postswelling Phase	Menstruation	Total
Average cycle	4	16	10	3	33
Short cycles					
11/38	0	16	12	1	29
4/39	1	12	13	1	27
3/42	2	14	10	2	28

* All numbers except dates refer to number of days.

was mated and each time conception occurred, so it is evident that these were ovulatory cycles despite the unusual characteristics of the first few days. In the other three cycles listed there was no opportunity for fertilization but if mating had occurred there is no reason to believe that conception would not have taken place. Since sexual swelling is produced only by a rise in estrogen level, it is necessary in these cases to assume increasing follicular activity concomitant with menstruation.

If one measures cycle length from the end of the preceding menstruation, the beginning of detumescence is earlier in these six cycles than in her average cycle or in most of her shorter cycles (see Table 3). The times calculated in this, the customary manner, are 20 days for the average cycle, 16, 13, and 16 days for the short cycles and 12, 12, 13, 11, 11 and 12 days for the six cycles under discussion. It is difficult to assign a definite length to cycles such as these. The interval between the cessation of menstruation and the end of the succeeding period of flow, although defined as the cycle length, is not necessarily correct in these instances. It is probable that considerable follicular growth and estrogen secretion had occurred by the time that cessation of luteal function had affected the endometrium for in artificial

cycles even when full estrogenic replacement therapy (as measured by complete tumescence of the sex skin) is instituted there is an appreciable delay before early tumescence can be noted. There is no information as to the exact size a follicle must attain before secretion of estrogen at a rate sufficient to affect the sex skins begins, nor is there any information as to how much time must elapse between the attainment of an adequate blood estrogen level and the beginning of tumescence. However, if swelling of the sex skin begins during menstruation or concomitant with the onset of flow there must have been an actively secreting follicle even earlier. In other words a cycle such as this begins (as indicated by the sex skin) before the preceding cycle (as dated by the cessation of flow) ends. The early occurrence of detumescence (and thus ovulation) cannot simply be considered to indicate a short cycle but on the contrary is a verification of the idea that the cycle began before the end of the preceding cycle. Observations such as this would be impossible in those species lacking a genital swelling and a similar condition may have existed in some of those numerous cases in the human where conception has occurred in spite of careful adherence to the contraceptive tenets of the rhythm theory.

INITIAL CYCLICAL EVENTS AT PUBERTY

The variations in the shape, size, and color of the swellings in the adult are to some measure foreshadowed in the adolescent animal. There are, however, considerable differences especially in the prominence of the clitoral area. At the very beginning of activity of the sex skin all that can be noticed in some individuals is a ventral prolongation of the labia, while in others the swelling consists of a small hemispherical mass involving both the labia and the anus. These swellings persist for varying lengths of time and are followed by variable periods of quiescent sex skin differing from animal to animal and from cycle to cycle for the same animal. The first menstruation has occurred as early as $4\frac{1}{2}$ months after the first appearance of swelling or as late as 3 years. As a general rule each successive swelling is slightly larger than the preceding one and full size may not be attained until after the menarche. The actual lengths of the different phases of the cycle in pubertal animals are discussed at some length by Young and Yerkes (1943) and need no further comment. The significance of the cyclical events at adolescence are discussed below in connection with the phenomenon of reinstitution of cycles after pregnancy.

CHANGES IN SEX SKIN DURING PREGNANCY

The sex skin varies in its activity during pregnancy. There may be as many as four or as few as no periods of swelling recorded as "maximum" during gestation (Nissen and Yerkes, 1943). Not only do the number of swellings vary but there seems to be little regularity in the time relations of such swellings as do occur. This is well demonstrated



FIG. 2. Changes in sex skin during two successive pregnancies in Bula.

in the two graphs of Figure 2 where the changes in the sex skin in two successive pregnancies in the same animal (Bula) are shown. The various steps correspond to the estimates made in the daily examination. The lack of correspondence between the two graphs is evident. For comparison the graphs of two non-pregnant cycles and of two average cycles are shown in Figure 3. Usually it is only during the first trimester of pregnancy that the sex skin attains a size comparable to the maximum of the normal cycle. Large swellings do occur in later phases of pregnancy but we have never seen large swellings in either of the last two trimesters unless there have been more or less regular

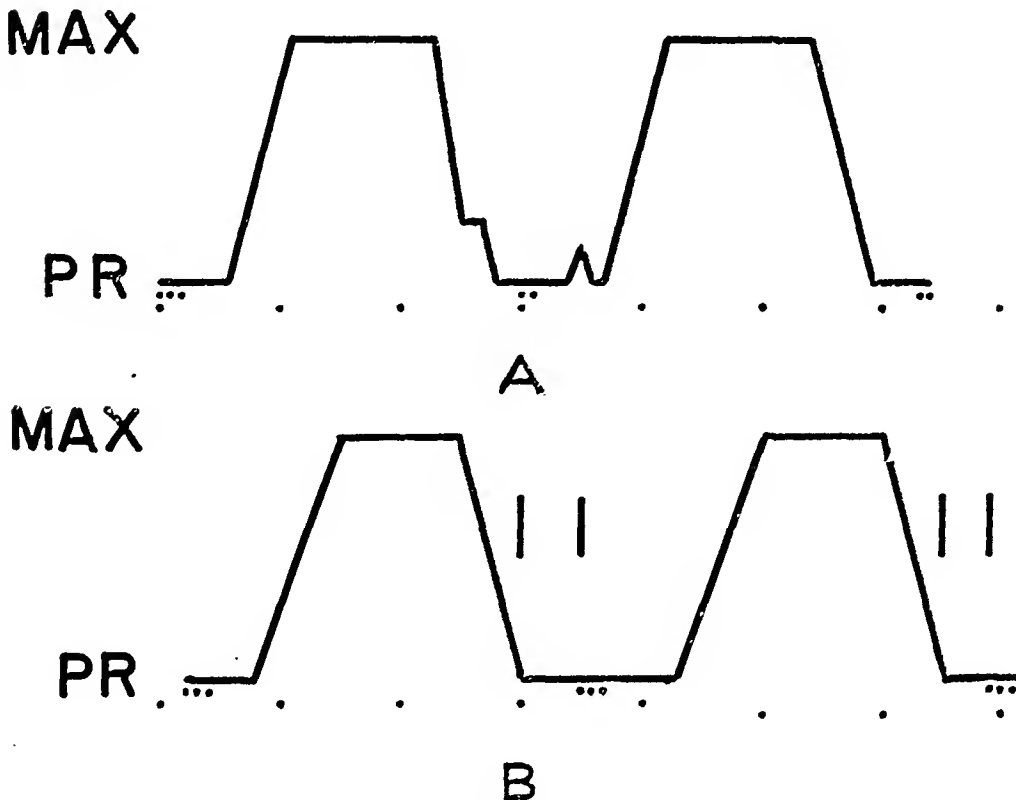


FIG. 3. A. Changes in sex skin in two successive cycles in Bula. B. Two average cycles. The period of premenstrual quiescence is indicated by the vertical lines.

episodes of tumescence during the first trimester. In addition there are many minor and irregular fluctuations both in the size and in the color of the non-pigmented portions of the genital area. There also seems to be a slow trend towards a deeper shade of rose but otherwise the changes seem unsystematic. These events are explicable in terms of some of the phenomena observed in artificial cycles. In connection with other work (Birch and Clark, 1946) it was necessary to give Nira, who was both ovariectomized and hysterectomized, a follicular hormone and a luteal hormone for a considerable period of time. The resultant changes in her sex skin are shown in Figure 4. The periods of treatment with anhydrohydroxyprogesterone (10 mg/day by mouth) are indicated by the solid line and those with alphaestradiol (2 mg/day by mouth) by the dotted line. In the first experiment after 29 days of treatment with the progestational hormone the estrogen was given in addition: The reverse was done in a second experi-

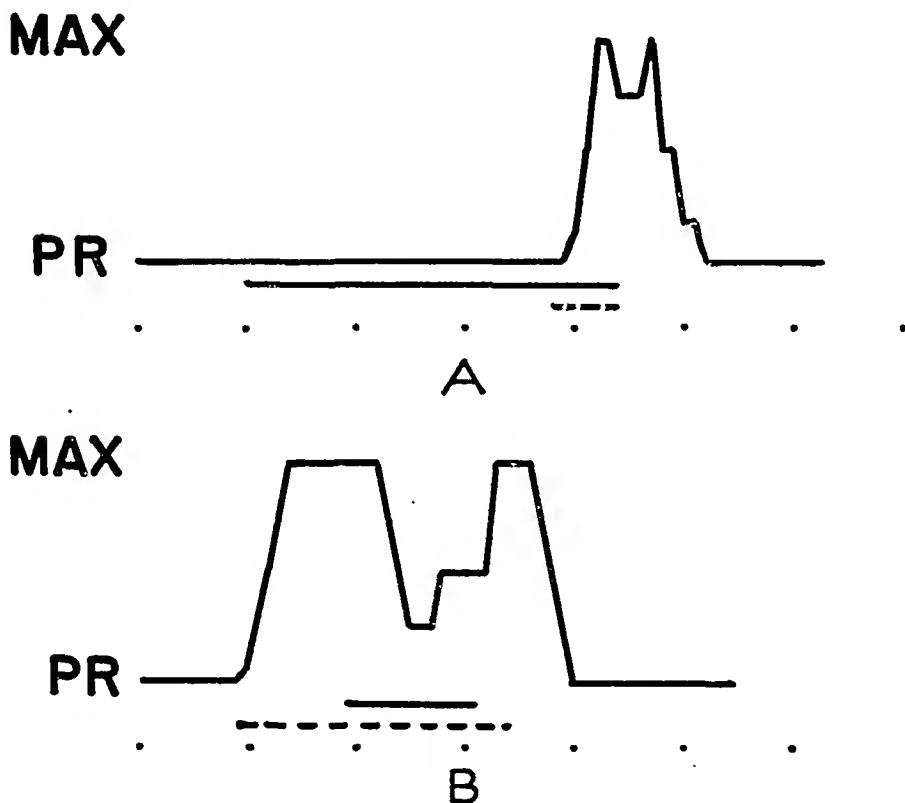


FIG. 4. Changes in sex skin during combined treatment with alphaestradiol and anhydrohydroxyprogesterone. A. Treatment with anhydrohydroxyprogesterone first followed by combined treatment. B. Treatment with alphaestradiol first followed by combined treatment. Period of treatment with anhydrohydroxyprogesterone indicated by solid line beneath graph. Period of treatment with alphaestradiol indicated by dashes beneath graph.

ment where, after a period of treatment with the estrogen, pregnenolone was also given. It will be seen (Figure 4) that the preliminary response of the sex skin was the same in both experiments. The latent periods between institution of estrogenic therapy and the onset of swelling and the time for attainment of complete tumescence were identical. It will be seen that in the second experiment the amount of pregnenolone was insufficient to produce total deturgescence and that after the swelling regressed to about $\frac{1}{4}$ of the maximum size returgescence occurred. For want of a better term one might call this an "escape" of sex skin from the inhibitory action of the progesen. The similarity with the condition during pregnancy may be only suggestive. The individual variation (see Figure 2) is so great that other factors must also be operative.

POSTPARTUM REINSTITUTION OF CYCLE

During lactation the sex skin is so quiescent that it appears similar to that of the castrate or pre-adolescent and thus bears slight resemblance to the resting sex skin of the late postswelling or of the preswelling phases in the normal animal. When cyclic activity begins anew the changes in configuration of the sex skin in successive cycles remind one of the adolescent but are more rapid. The swellings are at first small in size with progressive increases in subsequent cycles. However, when the baby is taken from the mother immediately or if it is stillborn the first swellings may approach full size. As Young and Yerkes (1943) have shown the lengths of the various phases of the cycles are also suggestive of the adolescent but again the changes in successive cycles are more rapid. Indeed if one compares the length of the postswelling phase preceding the first menstrual period after pregnancy with the first postswelling phase succeeding the first menstruation, the difference is statistically significant. That is, if the menstrual period marked "A" in the graphs of the normal cycles of Figure 3 was the one being considered then we would be comparing the period between the first parallel lines with the second. As determined by Fisher's "t" method (Snedecor, 1946) the difference between the first and second postswelling phases is significant to better than the 1% level. Where lactation occurred the "t" value is 3.503 and the requirement for the 1% level is 2.819. Where the baby was immediately taken from its mother or was stillborn the "t" value is 3.332 and the requirement for the 1% level is 2.787. A similar comparison of the postswelling phases directly before and after the menarche reveals no significant differences.

The increase in size of the swelling of the sex skin in successive cycles in the adolescent and after parturition, especially when there has been a long period of lactation, may be duplicated in the castrate female after the institution of replacement therapy. If only estrogen is given the swelling is small although the tension of the sex skin may

be maximal. When replacement therapy includes both estrogen and progesterone the size of each successive swelling rapidly increases to a maximum for the animal. Unless the dosage is sufficient, however, the swelling never becomes tense but only reaches a flabby state intermediate in size between the resting level and the maximum. In cycles where only estrogen was given the interval between the end of detumescence and the beginning of menstruation has never been greater than one day, but the interval becomes increasingly longer when both an estrogen and a progesten are given.

Young and Yerkes (1943) tentatively concluded that the short postswelling phases seen in adolescents and following parturition were similar and were probably due to a deficiency in production of progesterone. This is only partially true. It was stated earlier that in artificial cycles, where the dosage of progesterone was insufficient, complete detumescence failed to occur. In such cycles when both hormones are withdrawn simultaneously the time relations of the resultant catemnia are similar to those in cycles where estrogen alone was given. This is in marked contrast to the short post swelling phases, for in these there is usually complete regression of the sex skin before the onset of menstruation. In fact in the first cycles after the menarche in 14 animals the period of premenstrual quiescence averaged 2.4 days with a range of 0-6 days. In only four animals was the period less than two days. It is therefore evident that a deficiency of progesterone alone could not produce the short postswelling phases of the adolescent and postparturitional animals, and it is tentatively suggested that an estrogen deficiency may also be present. It should be emphasized, however, that we have not as yet duplicated such cycles artificially and that final decision should be held in abeyance. It will be interesting to check the endometrium during these periods for this may supply a partial answer to the problem of adolescent sterility.

COMPARISON OF CYCLE IN CHIMPANZEE AND IN THE CHACMA BABOON

During the preparation of this paper an important monograph on the sex cycle of the chacma baboon appeared (Gillman and Gilbert, 1946). In most particulars their data and ours are complementary. The cycle length in the chimpanzee, with a range of from 22 to 187 days and a mean of 37.28 ± 0.14 days (Young and Yerkes, 1943) is nearly as variable as in the baboon which has a range of from 17 to 238 days and a mean of 39.63 ± 0.7 days. In both the mode is much shorter being 33 days in the chimpanzee and 35 days in the baboon. It is probable that with skewed distributions such as these that the mode is the better measure of central tendency. The various subdivisions of the cycle in the baboon are slightly longer than in the chimpanzee. In both animals ovulation occurs before the onset of

deturgescence but much remains before the variation in delay of detumescence is explainable. In the details of the menarche the two species show some differences. In the chimpanzee repeated swelling and deturgescence occurs before the first menstrual flow while in some baboons the menarche is ushered in by an initial catemenia. Since we have data on only 17 chimpanzees the possibility exists that a bloody show may occasionally be the first external indication of beginning periodicity. The size of the sex skin swelling becomes increasingly larger during each of the first few cycles. This is also true of the initial swellings following parturition. During gestation the baboon also has rhythmical changes in the size of the sex skin swelling but the time relations are different. In the baboon the initial deturgescence following a fertile mating is slowly replaced by a slight swelling whose full size is not attained until about the 90th day. In contrast the first changes in the sex skin following a fertile mating except for the lack of menstruation, duplicate those of the normal cycle. In both time relations and size of the swelling there is initially no change from normal. Another difference between the chimpanzee and the baboon is in the time that elapses between hormone withdrawal and menstruation. In the baboon the interval between progesterone withdrawal and flow amounts to 2-5 days while for estrogen withdrawal the time is 10-15 days. By contrast in the chimpanzee the intervals between withdrawal and onset of flow are not noticeably different for progesterone withdrawal and estrogen withdrawal. The significance of these species differences is obscure.

CONCLUSIONS

Menstruation does not necessarily indicate a quiescent ovary. In fact there may be an actively secreting follicle at such a time.

There are probably considerable differences between the amounts of progesterone secreted in different cycles and in different animals.

Estrogen therapy alone may not be sufficient to produce a maximal size swelling in the chimpanzee but only maximum turgidity. Complete duplication of sex skin alterations requires estrogen and progesterone therapy.

Under long continued progesten therapy the sex skin becomes unresponsive to progesterone and swellings will occur with threshold amounts of estrogen. We have termed this the progesterone "escape" phenomenon.

The short postswelling phases in the adolescent and postpartum chimpanzee cannot be due to a deficiency in progesterone secretion alone. Another factor, probably inadequate estrogen secretion, seems to be involved.

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STUDIES ON THE BIOASSAY OF HORMONES

SKEWED RESPONSE OF THE CHICK OVIDUCT TO METHOXY BISDEHYDRO DOISYNOLIC ACID (MDDA)¹

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IN THE COURSE of our investigations on the role of vitamins in the action of estrogens on the chick oviduct it was observed that the responses of the synthetic estrogen methoxy bisdehydro doisynolic acid (MDDA) were unique since the intragroup variation in White Leghorn chicks was extraordinarily great. This communication presents the comparative oviduct responses to MDDA, estrone, estradiol benzoate, estradiol and stilbestrol in the White Leghorn chick and the oviduct responses to MDDA and stilbestrol on the Sex-linked chicks. It is the purpose of this paper to show that the skewed response of the oviduct occurs only for MDDA.

ANIMALS, METHODS, MATERIAL

The White Leghorn and Sex-linked pullets were obtained from Kerr Chickeries in Frenchtown, New Jersey. Treatment was begun on the fourth day of life and continued through the eighth day of life. The estrogens were dissolved in corn oil so that the total dose was contained in 0.5 c.c. of oil. The daily dose was contained in 0.1 c.c. of solution. Twenty-four hours after the last subcutaneous injection the animals were sacrificed with chloroform and body weight and oviduct weight was determined. All results are expressed as 100 times the ratio of the oviduct weight in milligrams to the body weight in grams.

The estrone, estradiol, estradiol benzoate, and methoxy bisdehydro doisynolic acid (MDDA) were generously supplied by Ciba Pharmaceutical Products, Inc. The stilbestrol was kindly supplied by Winthrop Chemical Co.

EXPERIMENTAL

The detailed data are presented in tables 1 and 2. Table 1 represents the influence of various estrogens on the oviducts of the White Leghorn chick. The mean oviduct ratios are presented along with the

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standard error (S.E.) and the range of the individual ratios within a given group.

In the control groups (Table 1) the range in individual ratios varied

TABLE 1. RESPONSE OF CHICK OVIDUCT TO ESTROGENS
(The estrogens were administered by subcutaneous injection to White Leghorn chicks.)

Estrogen	Total Concentration μg.	Number of Chicks	Oviduct Ratio	
			Mean ± S.E.	Range
None	0	17		
	0	15	9 ± 0.9	5-15
	0	24	11 ± 0.6	9-17
	0	21	11 ± 0.4	7-15
	0	25	11 ± 0.9	5-21
Estradiol	800	21	10 ± 0.6	6-12
	1600	23	69 ± 4	52-117
Estradiol Benzoate	100	44	105 ± 7	60-209
	400	23	22 ± 1	10-40
Estrone	40	18	123 ± 9	79-252
	1280	40	16 ± 0.9	9-23
Stilbestrol	25	28	75 ± 1.0	31-135
	50	24	29 ± 2	15-50
	100	54	55 ± 2	43-79
	200	53	71 ± 2	40-116
	400	74	143 ± 6	60-339
	800	19	270 ± 10	130-562
	1600	18	464 ± 29	278-746
	100	18	564 ± 27	329-698
	400	42	17 ± 3	7-57
	400	51	101 ± 28	13-735
Methoxy bisdehydro doisynolic acid (MDDA)	800	21	71 ± 15	19-459
	1600	45	36 ± 10	10-310
	1600	43	108 ± 33	14-945
			224 ± 43	24-958

by a factor of from 1.9 to 4.2 when the lowest and highest ratios are considered. The mean range factor for the five oil control groups was 2.6. Estradiol at a dose of 800 micrograms showed a ratio range

of from 52 to 117 or a factor of 2.3, while at 1600 micrograms the range was somewhat larger, 3.5. A range factor of 4.0 at 100 micrograms of estradiol benzoate and 3.2 at 400 micrograms of estradiol benzoate.

The range factors for estrone were 2.6 and 4.4 respectively for total doses of 40 and 1280 micrograms. For stilbestrol similar magnitudes of variations were found. The range factor varied from 1.8 at 50 micrograms to 5.6 at 200 micrograms. The mean range factor for the seven groups studies was 2.9.

With MDDA the range in oviduct ratios in the White Leghorn appears to be significantly increased. If we consider all eighteen groups other than those treated with MDDA we find a mean range factor of 3.1. For MDDA at a dose of 100 micrograms a ratio range factor of 8.2 was found. At 400 micrograms in two different experiments range factors of 57.5 and 24.1 were found. At 800 micrograms the range factor was 31.0 while at the highest dose studied, 1600 micrograms, the range factors of 67.5 and 39.9 were found. Over the dosage levels of 400, 800, and 1600 micrograms a mean range factor of 44 was found.

Table 2 presents a series of experiments in which Sex-linked chicks were used. Here the intragroup ratio variation for stilbestrol treated chicks was similar to that found for the White Leghorns. The mean ratio range factor for the four dose levels studied was 2.3 for the Sex-linked groups while the men ratio range factor for the White Leghorns was 2.9.

When MDDA was administered subcutaneously to the Sex-linked chicks only one animal in all five groups showed an aberrant response. This response was found at 750 micrograms where one ani-

TABLE 2. RESPONSE OF CHICK OVIDUCT TO ESTROGENS
(The estrogens were administered by subcutaneous injection to Sex-linked chicks.)

Estrogen	Total Concentration μg.	Number of Chicks	Oviduct Ratio	
			Mean ± S.E.	Range
Stilbestrol	50	23	43 ± 2	37-57
	100	20	84 ± 5	45-135
	200	20	174 ± 10	89-326
	400	17	290 ± 39	57-603
MDDA	200	28	21 ± 1	15-34
	400	28	22 ± 1	11-29
	750	35	34 ± 10	14-373
	1500	35	46 ± 3	17-100
	1600	28	38 ± 4	21-120

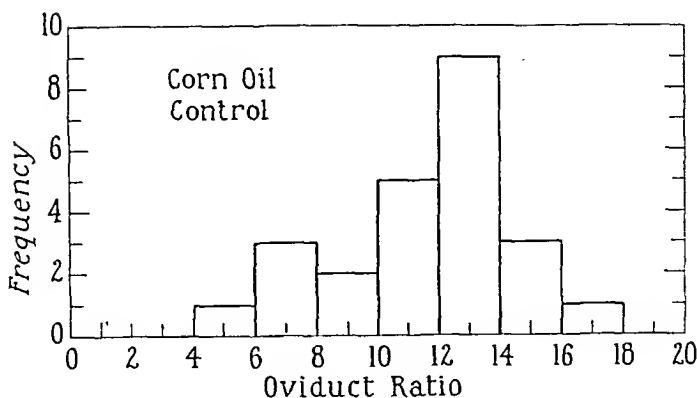


FIG. 1. Distribution of oviduct ratios of White Leghorn chicks treated only with the vehicle, corn oil.

mal showed a ratio of 474 while the remaining 34 chicks in the group showed a ratio range from 14 to 104. At 750 micrograms the ratio range factor was 26.6. However, at the other four concentrations the variations were quite normal. The following range factors were found: at 200 micrograms, 2.3; at 400 micrograms, 2.6; at 1500 micrograms 5.9; at 1600 micrograms, 5.7.

Figures 1 through 3 illustrate graphically the skewness of the oviduct response in White Leghorn chicks to MDDA as compared to that of control and estrone treated animals.

DISCUSSION AND CONCLUSIONS

It is apparent from the data that the response of the White Leghorn chick oviduct to MDDA is unique. When Sex-linked chicks, a cross between Plymouth Rocks and Rhode Island Reds, were employed the unusual skewed response was not found. The reason for the abnormal response of the White Leghorn chick oviduct to MDDA

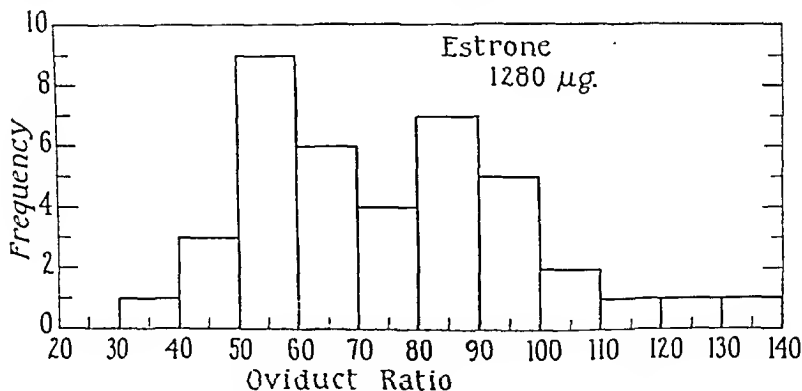


FIG. 2. Distribution of oviduct ratios of White Leghorn chicks treated with 1280 μ g. of estrone.

group was given 50 gamma of growth hormone daily while each of those in the other group received 150 gamma daily. After 28 days of injection at this level, the daily dose was doubled for each group and was continued at the higher level for 22 more days. Next, was a 23-day control period in which no injection was given. After the second control period growth hormone was again administered but at the original dose level. This was continued for 18 days and then a final control period of 12 days was carried out.

RESULTS AND COMMENT

The data are presented graphically in Figs. 1 and 2 in which are shown the mean daily urinary nitrogen excretions, together with the daily standard deviations of the means. It will be seen that the initial administration of hormone at both the 50 and the 150 gamma dose levels produced nitrogen retention and body weight gain but that

The Effect of Injection of Growth Hormone for Seven Weeks on
Body Weight and Daily Urinary Nitrogen Excretion of Adult Female Rats

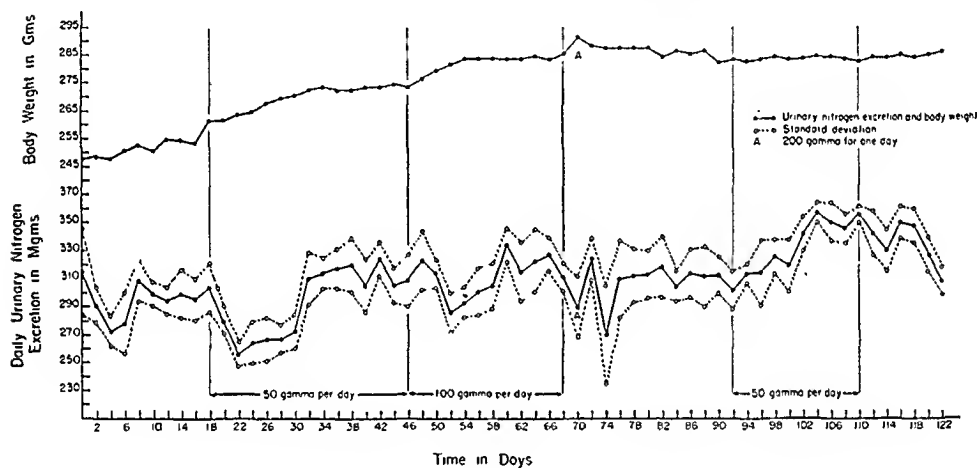


FIG. 1.

The Effect of Injection of Growth Hormone for Seven Weeks on
Body Weight and Daily Urinary Nitrogen Excretion of Adult Female Rats

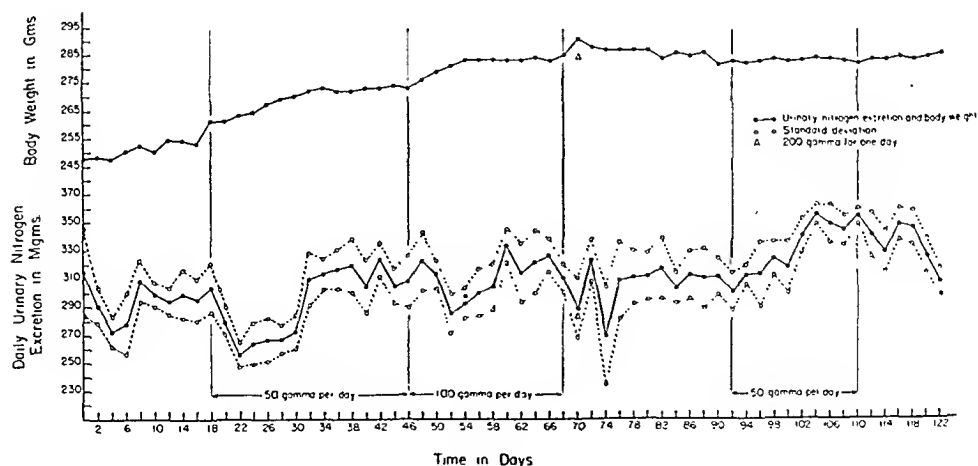


FIG. 2.

these effects did not persist. The body weight tended to plateau again and the urinary nitrogen excretion returned to its original pre-injection level. When the amount of hormone given was doubled, nitrogen retention and body weight gain was produced once more. But again these effects were evident only during the first part of the period of hormone administration.

When the growth hormone administration was discontinued, the animals which had been receiving 300 gamma of hormone per day lost weight and had a daily urinary nitrogen excretion appreciably in excess of that during the pre-injection control period. The body weight and nitrogen excretion of the animals which had received previously 100 gamma of hormone per day remained stable throughout the second control period. When growth hormone was readministered at the initial dose level no effect was produced by the 50 gamma dose. In fact, the urinary nitrogen excretion increased during this period of treatment. The readministration of hormone at the 150 gamma level again produced a transient nitrogen retention and body weight gain. These observations are not in conflict with the previously reported fact (Evans, Simpson, and Li, 1948) that growth hormone will produce continued growth in normal rats if the dose level is progressively increased. There are two likely possibilities as to the explanation of the lack of persistence of the effect of a continual administration of small doses of hormone. First, the supply of exogenous hormone may have suppressed the endogenous secretion of growth hormone by the animals own pituitary. If this were the explanation, repeated small doses of hormone should have a constant effect in hypophysectomized rats. Second, there may have developed anti-growth substances in the treated animals. Proof of this hypothesis would be furnished by the in vivo antagonism by the serum of treated animals of growth hormone action. This has not been demonstrated.

SUMMARY

Urinary nitrogen excretion and body weight of two groups of 6 animals each of "plateaued" normal female rats were followed for 122 days. Initially growth hormone was administered at a dose of 50 and 150 gamma per day for 28 days. Both dose levels produced nitrogen retention and body weight gain only during the first half of the injection period. The original dose was doubled for an additional 22-day period and again a transient period of nitrogen retention and body weight gain resulted. After an additional 23-day control period, the readministration of 50 gamma of hormone was without effect, while the readministration of 150 gamma of hormone again produced a transient period of nitrogen retention and body weight gain.

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EFFECTS OF PITUITARY ADRENOCORTICOTROPIC HORMONE ON THE INTACT RAT, WITH SPECIAL REFERENCE TO CYTOCHEMICAL CHANGES IN THE ADRENAL CORTEX^{1,2}

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THE AVAILABILITY of adequate quantities of pituitary adrenocorticotrophic hormone provided the opportunity for testing the hypothesis that, in the rat, the secretion of only the cortical "sugar" factors is under pituitary control (Swann, 1940; Sarason, 1943; Deane and Greep, 1946). Morphological evidence obtained thus far suggests that these hormones (the 11-oxygenated corticosteroids) are secreted by the zona fasciculata of the adrenal cortex. This zone atrophies following hypophysectomy, but may be restored to normal by the administration of adrenocorticotropin (Simpson, Evans and Li, 1943). Furthermore, the zone enlarges and appears hyperactive when animals with intact pituitaries are subjected to physiological demands for increased gluconeogenesis (Dalton, Mitchell, Jones and Peters, 1944; Deane and McKibbin, 1946). The salt-regulating hormones of the adrenal do not seem to be under pituitary control in the rat. Salt balance is not seriously disturbed following hypophysectomy. Moreover, retention of sodium chloride does not follow the administration of adrenocorticotropin (Ingle, Li and Evans, 1946; Ingle, Prestrud, Li and Evans, 1947). Hormones of the salt-regulating type appear to be secreted principally by the outermost zone of the cortex, the glomerulosa. This zone atrophies and becomes inactive when desoxycorticosterone acetate is administered over a prolonged period (Greep and Deane, 1947). On the other hand, the glomerulosa enlarges and appears active in rats relatively deficient in sodium; the latter alteration develops in hypophysectomized as well as intact rats (Deane, Shaw and Greep, 1948).

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The present investigation represents an attempt to discover whether or not the secretion of both the "salt" and "sugar" hormones of the adrenal cortex is stimulated by adrenocorticotropin in the rat. Following the administration of hormone, changes in the size of the adrenals and in the two major zones of the cortex were studied. These changes were correlated with alterations in the weight of the thymus and in the levels of chemically-determined steroid in the adrenal, of nitrogen and electrolyte excretion, and of glycogen in the liver. The effects of both short-term and prolonged administration of the hormone were investigated.

MATERIAL AND METHODS

Experimental plan

1. *Short-term experiments.* Experiments lasting 24 hours were carried out on groups of male rats of the Sprague-Dawley strain weighing between 60 and 250 grams. The animals were fed a diet of Purina dog chow until 8:30 A.M. of the day of the experiment, when they were weighed and transferred to individual cages. No food was supplied, but distilled water was provided *ad libitum*. Five mg per cc of adrenocorticotropin (Armour, sample #21B⁵) was dissolved in an alkalized 1 per cent saline solution, and the fasting rats received subcutaneous injections of selected amounts of the solution at 6-hour intervals during the succeeding 18 hours. As will be described below, the experiments were designed to test the effect of dosage and duration of treatment. Six hours after the final injection (24 hours total fast), the animals were weighed, killed by a blow on the head and autopsied. As controls, some rats were fasted but otherwise untreated; some received 0.5 cc of a 1 per cent sodium chloride solution on the same time schedule; others received pituitrin in amounts comparable to that contaminating the adrenocorticotropin preparation.⁵

The 24-hour output of urine was collected free from stools in a dish covered with wire-mesh. At the end of the day the pan and wire-mesh flooring were thoroughly flushed with water. An aliquot of this solution was preserved with thymol in the refrigerator for chemical analysis.

2. *Long-term experiment.* A long-term experiment lasting 12 days was carried out on a group of 19 male rats of the Sprague-Dawley strain weighing about 225 grams. These animals were transferred to a medium-carbohydrate diet (Table 1) which was given by stomach tube. The diet was similar to that used by Reinecke, Ball and Samuels (1939). In order to avoid "food shock," the rats were given small quantities of the mixture for the first day,

⁵ Two samples of adrenocorticotropin prepared from hog pituitaries were used. The hormone was purified by isoelectric precipitation in the cold and was claimed to contain negligible amounts of other anterior pituitary hormones. Sample #21B contained 0.12 units posterior pituitary oxytocic and pressor activity per mg. It was only slightly soluble in a 1 per cent solution of sodium chloride but could be dissolved in saline solution alkalized to pH 8. Sample #13-10-6 contained 0.07 units oxytocic and pressor activity per mg. It was readily soluble in a 1 per cent saline solution. Relative potencies of these two samples as compared to Armour's Standard Preparation (La-I-A), which shows a measurable decrease in adrenal ascorbic acid with as little as 2 μ g of hormone by the test of Sayers and Sayers (1945), were as follows: #21B, 102 \pm 8%; #13-10-6, 83 \pm 9%.

TABLE 1. COMPOSITION OF THE MEDIUM CARBOHYDRATE DIET FOR TUBE-FEEDING THE RATS ON THE LONG-TERM EXPERIMENT. THE ANIMALS RECEIVED 24 CC OF THE MIXTURE DAILY.

Constituent	Amount
Cascin (Vitamin Test, Chagrin Falls)	160 g
Starch	300 g
Dextrin	150 g
Sucrose	140 g
Cellu flour (Chicago Dietetic Supply Co.)	120 g
Dried yeast (Anheuser-Busch)	100 g
Salt mixture (Phillips and Hart IV)	40 g
Vitamin K (Menadione, Merck)	100 mg
Mazola oil	200 cc
Cod liver oil	10 cc
Wheat germ oil	10 cc
Water, to make a total of	2000 cc

then larger amounts, so that they attained the full amount of 12 cc per feeding 2 days before the beginning of injections. Two feedings were given a day, one at 8:30 AM and one at 4:30 PM. The two feedings supplied about 7 g of available carbohydrate. Distilled water was provided *ad libitum*.

Adrenocorticotropin sample #13-10-6⁵ was used for this experiment. The hormone was dissolved in a 1 per cent solution of sodium chloride at a concentration of 8 mg per cc. This solution was made up fresh daily and kept refrigerated. Subcutaneous injections of 0.5 cc were given to 13 of the rats at 8-hour intervals, starting at 8:00 AM on the first day of the experiment. The injections provided 12 mg hormone daily. For control purposes, 0.5 cc of a 1 per cent solution of sodium chloride was given to each of the other 6 rats, on the same time schedule. Urine was collected every 48 hours. Four experimental and 2 control rats were killed on the 4th, 8th, and 12th days of the experiment, respectively, after fasting the previous 24 hours.

Analytical methods

At autopsy, the liver, the two adrenal glands and the thymus gland were carefully weighed.

A piece of the right lobe of the liver was excised immediately for determination of glycogen. For chemical analysis, the piece was placed in a tared vessel containing 30 per cent potassium hydroxide, and the glycogen content was measured by a modification of the technique of Good, Kramer and Somogyi (1933). The Somogyi (1945) method was used for determining the reducing sugar after hydrolysis. In one experiment the quantity of glycogen in the liver was estimated visually in histochemical preparations. For this, the piece of liver was fixed in chilled picro-alcohol-formalin (Rossman's fluid), embedded in paraffin and sectioned at 5 μ . Glycogen was visualized by the Bauer-Feulgen reaction (Bensley, 1939).

The right adrenal gland was macerated in a mixture of equal parts of acetone and alcohol for the determination of total steroid ("cholesterol"), according to the method of Sperry and Schoenheimer (Sperry, 1938). The left adrenal gland was fixed in a 10 per cent neutralized formalin solution for the cytochemical identification of ketosteroids in the cortex. After 48 hours' fixation, the gland was washed in running water for an hour and then sectioned on a freezing microtome at 15 μ . One section was stained with sudan

IV and Harris' hematoxylin, another with sudan black B, and a third by the Schiff "plasma" method; these were mounted in glycerin jelly. Two sections were mounted unstained in glycerin; one of these was untreated and the other had been extracted for one-half hour in acetone at room temperature. The latter two sections were examined under the polarizing and fluorescence microscopes for acetone-soluble materials exhibiting birefringence and autofluorescence. This variety of methods is believed to identify ketosteroids under the conditions employed, since no other known type of compound is Schiff-positive, birefringent and autofluorescent as well as acetone-soluble and sudanophilic (Dempsey and Wislocki, 1946). The following urine analyses were made. The presence of glucose was ascertained by the Benedict reaction. Total nitrogen was determined by the method of Daly (1933). The amount of chloride was obtained by the technique of Schales and Schales (1941), with the precautions noted by Asper, Schales and Schales (1947). Total phosphorus was measured by the method of Allen (1940). The quantities of sodium and potassium were determined on a flame photometer with an internal lithium standard⁶ (Berry, Chappell and Barnes, 1946).

OBSERVATIONS

A. Short-term experiments

Four different types of 24-hour experiment were undertaken to study the short-term effects of the adrenocorticotrophic hormone. The experiments were designed to test the effect of dosage level (Exps. a and b) and of duration of treatment (Exps. c and d). The adrenal and thymus glands were weighed, and the total amounts of adrenal steroid, liver glycogen, and urinary nitrogen and chloride were determined, as well as the quantity and distribution of ketosteroids in the adrenal cortex in cytochemical preparations.

Fasting, handling and injecting rats lead to a variable "alarm" reaction. Therefore all of the control animals were fasted. In addition, some were injected with a solution of sodium chloride in the same volume as the hormone solution on similar time schedules. Moreover, since the samples of hormone employed contained up to 0.12 units of posterior pituitary oxytocic or pressor activity per mg, a third group of control rats was injected with pituitrin in amounts comparable to that received by the experimental animals. Identical tests were applied to the control rats.

Control experiment

In the basic control experiment, fasting rats received 3 injections of 0.5 cc of a 1 per cent saline solution or of 0.28 units pituitrin (Parke-Davis) at 8-hour intervals (0, 8 and 16 hours). Eight hours after the last injection the animals were killed. The figures for liver glycogen, urinary nitrogen and chloride, thymus adrenal weight and total ad-

⁶ We are indebted to Dr. William Wallace, Boston Children's Hospital, for carrying out the sodium and potassium determinations.

renal steroid for these rats are presented in Table 2. No difference between the two groups was observed except in chloride excretion, which was higher in the animals receiving pituitrin.

The cytochemical preparations of the adrenal glands of both of these groups of rats resembled those of uninjected, fasted controls. All displayed the normal distribution of ketosteroids in the cortex (see Deane and Greep, 1946; Deane and Shaw, 1947; Greep and Deane 1947). That is, sudanophilic droplets, which were autofluorescent and Schiff-positive and contained birefringent particles, were crowded in

TABLE 2. GROSS ANATOMICAL AND CHEMICAL DATA FOR THE CONTROL RATS. FASTED RATS INJECTED WITH 0.5 CC 1 PER CENT SODIUM CHLORIDE OR 0.28 UNITS PITUITRIN 3 TIMES AT 8-HOUR INTERVALS AND KILLED 8 HOURS AFTER LAST INJECTION (TOTAL, 24 HOURS)

Treatment	No. rats	Wt., g	Total liver glycogen, mg	Urine		Thymus wt., mg/100 g b. wt.	Adrenal glands	
				N, mg	Cl, mEq		wt., mg/100 g b. wt.	steroid, mg/100 mg gland
Saline	2	237	8	87	.24	155	22.7	4.70
Pituitrin	2	238	8	73	.62	212	21.3	4.70

the cells of the zona glomerulosa, absent from a transitional zone between the glomerulosa and fasciculata, abundantly present in the outer fasciculata, and virtually absent from the juxtamedullary region (Figs. 4, 6 and 8). The birefringent particles in both the glomerulosa and outer fasciculata were characteristically mixed in size, some being fine and some coarse.

It can be concluded from these chemical and cytochemical data that neither the injection of pituitrin in amounts comparable to that contaminating the adrenocorticotropin samples nor the injection of saline induced any significant alteration in the adrenal cortex. With comparable amounts of posterior pituitary hormone, Sayers, Sayers, Fry, White and Long (1944) likewise failed to obtain adrenal hypertrophy or a fall in adrenal "cholesterol." The chloruretic effect of pituitrin may have accounted for the increased chloride excretion in the group of rats receiving this hormone.

Effects of adrenocorticotropic hormone (sample #21B)

a. *Four injections of 2.5 mg hormone.* Ten mg adrenocorticotropin was administered to fasting rats in 4 equal doses of 2.5 mg every 6 hours (0, 6, 12 and 18 hours). The animals were killed 6 hours after the last injection. The following changes from the control condition were observed. A considerable deposition of glycogen occurred in the liver (equivalent to more than 1000 mg per 100 g wet liver). Nitrogen and chloride excretion both increased. The weight of the paired adrenal glands increased, whereas total adrenal steroid decreased appreciably, as did thymus weight (Table 3).

As judged by the cytochemical preparations, the ketosteroids of the zona fasciculata declined sharply in all of the animals receiving 10 mg hormone over a period of 24 hours. Sudanophilic droplets disappeared almost entirely from the inner part of the fasciculata and became more irregular in distribution peripherally (Fig. 5). Birefringent particles became sparse, and the remaining ones were all fine, rather than mixed in size (Fig. 7). Although the number of Schiff-positive and autofluorescent droplets declined, the intensity of these reactions in the residual droplets remained intense. Other changes in

TABLE 3. GROSS ANATOMICAL AND CHEMICAL DATA FOR FASTED RATS RECEIVING 10 MG ADRENOCORTICOTROPIN IN 4 DOSES OF 2.5 MG AT 6-HOUR INTERVALS. ANIMALS KILLED 6 HOURS AFTER LAST INJECTION (TOTAL, 24 HOURS). THE FIRST GROUP OF CONTROL RATS RECEIVED 0.5 CC 1 PER CENT SODIUM CHLORIDE PER INJECTION, THE SECOND GROUP WAS UNTREATED

Treatment	No. rats	Wt., g	Total liver glycogen, mg	Urine N, mg	Urine Cl, mEq	Thymus wt., mg/100 g b. wt.	Adrenal glands wt., mg/100 g b. wt.	steroid, mg/100 mg gland
Saline Hormone	3	233	18	173	.68	—	20	4.38
	3	236	174	192	1.96	—	32	3.06
None Hormone	2	194	26	—	—	162	20.9	3.20
	2	194	119	—	—	117	22.3	2.89

these adrenals included a widening of the whole fasciculata, a broadening of the transitional zone, and the development of large watery vacuoles in the cells of the outer fasciculata (Fig. 5). The zona glomerulosa, however, was unaffected by hormone injection in all of the short-term experiments (Figs. 5, 7, 9, 10, and 11).

In conclusion, these chemical and cytochemical results suggest the release of steroid hormones by the fasciculata under the influence of adrenocorticotropin. The decrease in chemically-determined steroid in the gland was accompanied by a reduction of ketosteroid droplets in the fasciculata. Moreover, there were other signs of increased secretory activity by the cells in the fasciculata. For example, the birefringent particles became finer, and the presence of uniformly fine birefringent material has repeatedly been shown to accompany hormone release (Weaver and Nelson, 1943; Deane and Shaw, 1947; Deane and Greep, 1947). The maintenance of the Schiff reaction in the residual droplets suggests the continued generation of carbonyl groups, which would be expected to accompany the formation of active hormones from precursors such as cholesterol (Bloch, 1945). Watery vacuolation of the cells in the outer fasciculata has also been observed frequently in the response to physiological stress (see Deane and Shaw, 1947, Fig. 2). The various metabolic changes observed, i.e., thymus involution, increased liver glycogen and the increase in urinary nitrogen and chloride, all point to a more rapid re-

lease of 11-oxygenated corticosteroids (Dougherty and White, 1945; Ingle and Thorn, 1941; Thorn, Engel and Lewis, 1941), although it is possible that some of the rise in chloride excretion may have resulted from the pituitrin contaminating the hormone product (Table 2). The changes noted simulate those resulting from a typical "alarm" reaction to physiological stress (Selye, 1946; Deane and Shaw, 1947).

In a recent review, Sayers and Sayers (1948) have indicated by diagrams that reduction of glomerulosa lipid follows adrenocorticotropin administration or physiological stress. However, in the one photomicrograph presented (Fig. 1b), the fasciculata appears exhausted of lipid, while the glomerulosa retains its full complement. No evidence was given for increased secretion of salt-regulating hormones.

b. *Four injections of smaller amounts of hormone.* To obtain an indication of the amount of adrenocorticotropin required to produce adrenal stimulation and the associated metabolic changes, two groups of fasting rats were injected with doses smaller than 2.5 mg at 6-hour intervals (0, 6, 12 and 18 hours). The animals were killed 6 hours after the last injection. These rats were smaller than in Exp. a, the first group weighing about 60 grams and the second 90 grams. For the first group, the amount of glycogen in the liver was estimated visually in preparations stained by the Bauer-Feulgen technique.

In the rats weighing 60 grams (Table 4A), adrenal weight increased with as little as 0.63 mg hormone per injection (total, 2.5 mg).

TABLE 4. GROSS ANATOMICAL AND HISTOCHEMICAL DATA FOR FASTED RATS RECEIVING DIFFERENT DOSES OF ADRENOCORTICOTROPIN 4 TIMES AT 6-HOUR INTERVALS. ANIMALS KILLED 6 HOURS AFTER THE LAST INJECTION (TOTAL, 24 HOURS).

A.					
Treatment	No. rats	Wt., g	Liver glycogen* (visual judgment)	Thymus wt., mg/100 g b. wt	Adrenal glands wt., mg/100 g b. wt
None	1	75	+	253	22.6
Saline, 0.5 cc	1	66	+	260	24.7
Hormone, 0.63 mg	1	62	+	258	30.6
Hormone, 1.25 mg	1	64	+	225	30.3
Hormone, 2.5 mg	1	58	+++	131	31.2

B.					
Treatment	No. rats	Wt., g	Thymus wt., mg/100 g b. wt.	Adrenal glands wt., mg/100 g b. wt.	Adrenal glands steroid mg/100 mg. gland
Saline, 0.25 cc	3	89	345	32.6	2.92
Hormone, 0.6 mg	3	86	319	35.5	2.20
Hormone, 1.25 mg	3	92	304	30.5	1.76
Hormone, 1.9 mg	3	88	296	35.0	1.54
Hormone, 2.4 mg	3	81	288	37.5	0.94

* Liver glycogen was evaluated on a basis of 0-4+. + indicates that only scattered cells contained glycogen; +++ indicates that considerable glycogen was present in the central part of the lobule.

Thymus involution began with the dose of 1.25 mg (total, 5 mg). Increased deposition of glycogen in the liver occurred only with the 2.5 mg dose (total 10 mg). In the rats weighing 90 grams (Table 4B), adrenal steroids fell progressively with increasing dosage. Thymus involution was significant with 1.25 mg per injection. However, for this group the data do not clearly indicate at what dosage-level adrenal hypertrophy occurred.

The cytochemical preparations of the adrenals of these rats revealed a significant decrease of ketosteroids from the fasciculata with a total dosage of 5 mg hormone in the first group and 7.6 mg in the second. With 10 mg the effect was more pronounced in these small rats than in the larger animals used in Exp. a (compare Figs. 7 and 9). With smaller doses (5-7.6 mg), the alterations were similar to, but less extreme than, those obtained with 10 mg. That is, sudanophilic droplets disappeared from the innermost part of the fasciculata with the small doses and more peripherally with larger ones. Birefringent particles became fine with the small doses and less numerous with larger ones. Particles had completely disappeared from the fasciculata with a total dose of 10 mg hormone (Fig. 9). Again, the Schiff reaction and autofluorescence of any residual droplets remained intense.

This experiment suggests that adrenal changes following treatment with adrenocorticotropin (hypertrophy of the gland and a decrease in its steroid content, both chemically and cytochemically) may occur without any significant deposition of glycogen in the liver. Only larger doses, probably resulting in the secretion of much more hormone, produce this effect. Moreover, a comparison of these results with those for larger rats (Table 3) indicates that 10 mg hormone produces more extreme effects in small rats (60-90 g) than in large ones (195-230 g).

c. *Injection of 10 mg hormone in periods shorter than 24 hours.* To determine whether any difference exists in the time of reaction to the administration of adrenocorticotropin by the cortex and by the rest of the body, rats were killed 6, 12, 18 and 24 hours after the beginning of treatment. In this experiment, all of the rats were starved 24 hours, and all received a total of 10 mg hormone. The first group received a single injection of 10 mg on the 18th hour of the fast; the second, 2 injections of 5 mg at 12 and 18 hours; the third, 3 injections of 3.3 mg at 6, 12 and 18 hours; and a final group, 4 injections of 2.5 mg at 0, 6, 12 and 18 hours. All animals were killed at the end of the 24-hour fast.

Inspection of Table 5 reveals that total adrenal steroid decreased within 6 hours and reached the lowest level at 12 hours. With more prolonged treatment, there was a gradual return toward the original proportion. Adrenal weight, on the other hand, reached its maximum only with treatment of 18 hours, after an initial fall. Thymus involution was detectable at 6 hours, the deposition of glycogen in the liver only at 18-24 hours.

TABLE 5. GROSS ANATOMICAL AND CHEMICAL DATA FOR FASTED RATS RECEIVING A TOTAL OF 10 MG ADRENOCORTICOTROPIN IN 1, 2, 3 OR 4 INJECTIONS AND KILLED 6 HOURS AFTER THE FINAL INJECTION (TOTAL STARVATION, 24 HOURS).

Treatment	No. rats	Wt., g	Total liver glycogen, mg	Thymus wt., mg/100 g b. wt.	Adrenal glands wt., mg/100 g b. wt.	steroid, mg/100 mg gland
None	2	217	19	162	17.3	3.33
1 inj. (10 mg)	2	269	10	92	13.7	1.69
2 inj. (5 mg)	2	241	27	129	17.3	1.52
3 inj. (3.3 mg)	2	234	50	103	20.8	1.72
4 inj. (2.5 mg)	2	247	121	52	18.0	2.10

A study of the cytochemical preparations of these adrenal cortices revealed the following changes in the zona fasciculata. The adrenals of animals receiving individual injections larger than 3.3 mg were

EXPLANATION OF PLATE 1

All photomicrographs are of rat adrenal glands that were fixed in 10 per cent neutralized formalin for over 2 days, washed thoroughly and sectioned at 15μ on the freezing microtome. The borders of the cortex have been drawn in on the photographs of birefringence preparations. All figures on this plate $\times 90$.

FIGS. 4-7. Experiment a, in which fasting rats received 10 mg adrenocorticotropin in 4 divided doses at 6-hour intervals. Animals killed at end of 24 hours.

FIG. 4. Control rat, 4 injections of 0.5 cc saline. Sudan IV and hematoxylin. Sudanophilic droplets are densely packed in the cells of the zona glomerulosa and the outer fasciculata, with a lipid-poor transitional zone between. Relatively little lipid occurs in the juxtamedullary region.

FIG. 5. Experimental rat, 4 injections of 2.5 mg hormone. Sudan IV and hematoxylin. The fasciculata is considerably broader than in Fig. 1. The glomerulosa contains the usual complement of lipid. The transitional zone is broadened. Lipid has entirely disappeared from the inner fasciculata and is less uniformly distributed in the outer fasciculata. The cells of the fasciculata appear considerably swollen in comparison to normal, and many display watery vacuoles.

FIG. 6. Control rat, uninjected. Birefringence preparation. A mixture of fine and coarse birefringent particles occurs in the glomerulosa and in the outer three-quarters of the fasciculata.

FIG. 7. Experimental rat, 4 injections of 2.5 mg hormone. Birefringence preparation. The fasciculata appears broadened. The birefringent particles in the glomerulosa are unaltered, while those in the fasciculata are sparser, finer and duller than in Fig. 6.

FIGS. 8 and 9. Experiment b, in which fasting small rats were injected with 10 mg adrenocorticotropin or less in 4 divided doses at 6-hour intervals. Animals killed at end of 24 hours. Birefringence preparations.

FIG. 8. Control rat, uninjected. Birefringent material occurs in the glomerulosa and throughout the fasciculata.

FIG. 9. Experimental rat, 4 injections of 2.5 mg hormone. The glomerulosa is unaffected. All birefringent material has disappeared from the fasciculata. More pronounced depletion than in a larger animal receiving same dose (Fig. 7).

FIG. 10. Experiment c, in which fasting rats received a total of 10 mg. adrenocorticotropin in varying number of injections. Rat receiving 10 mg in 1 injection and killed 6 hours later. Birefringence preparation. The transitional zone appears much broader than in Fig. 6. The fasciculata is broad; fine birefringent material has disappeared entirely; and all birefringent material has gone from the inner portion.

FIG. 11. Experiment d, in which fasting rats received 2.5 mg adrenocorticotropin for a different number of times. Rat receiving 3 doses (total 7.5 mg) and killed 18 hours after the first. Birefringence preparation. The glomerulosa retains its birefringent material. Only sparse, small particles remain in the fasciculata. Less material present than with treatment for 24 hours (Fig. 7).

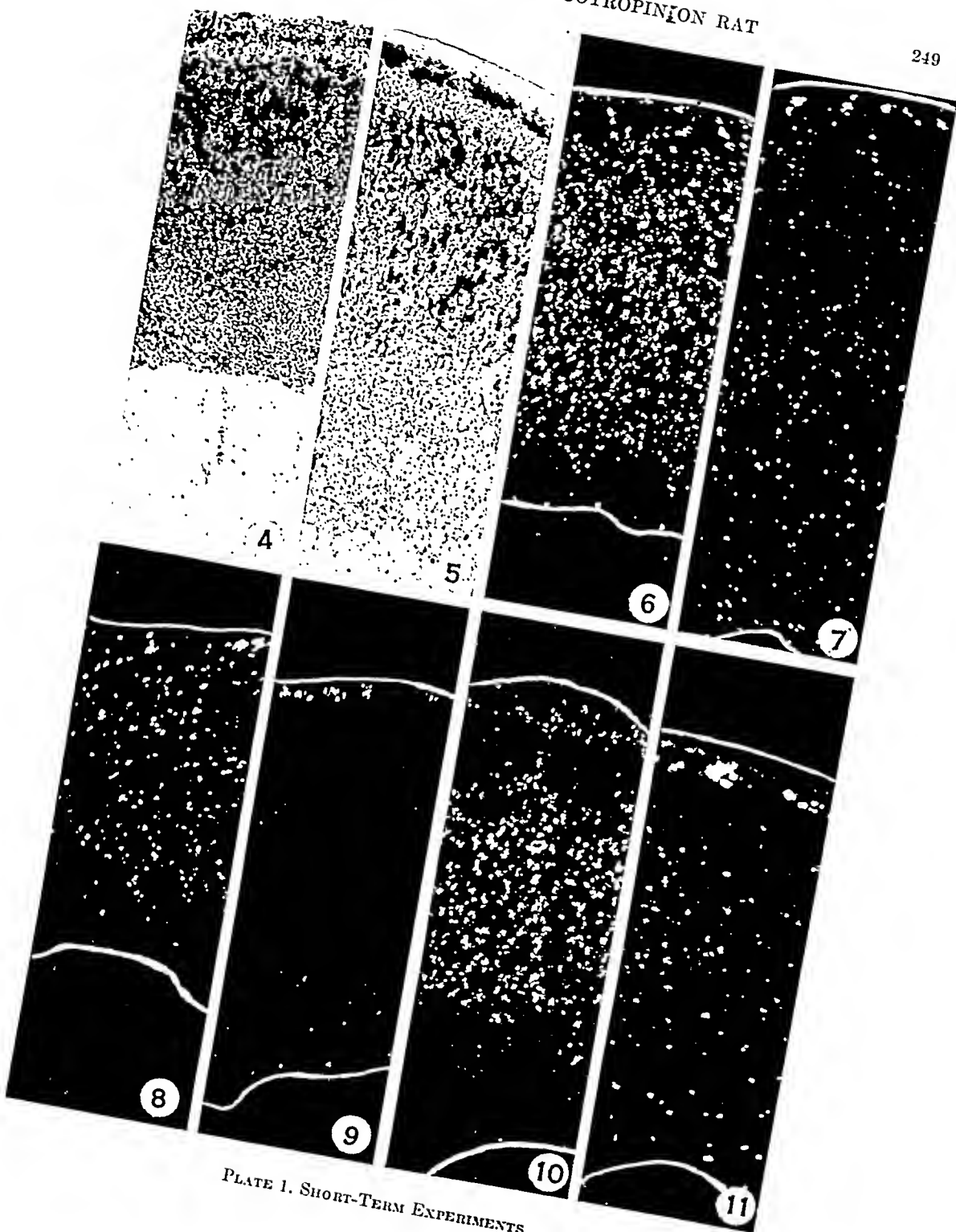


PLATE 1. SHORT-TERM EXPERIMENTS

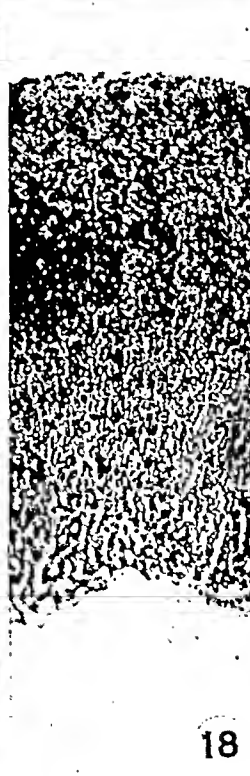
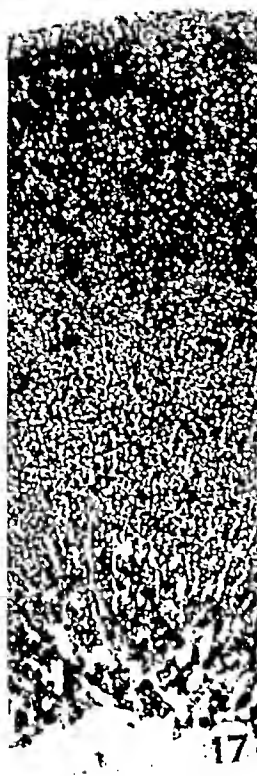
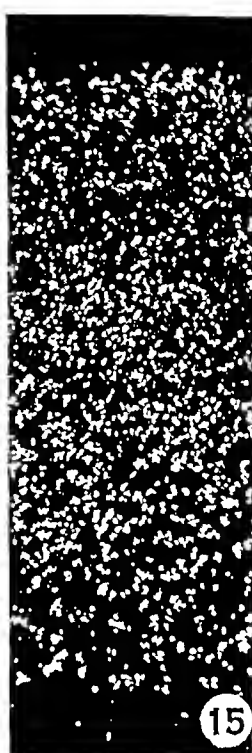
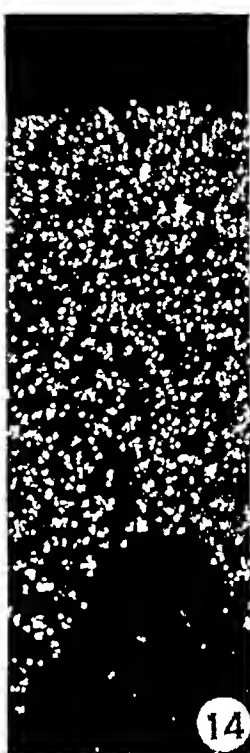
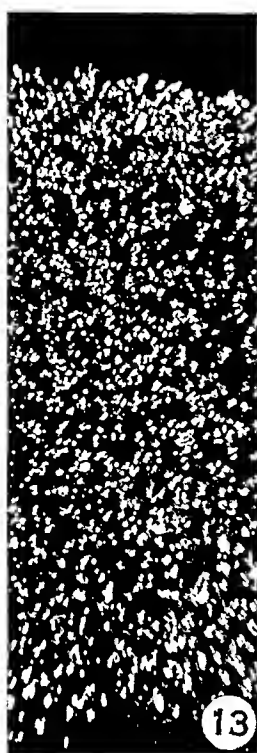
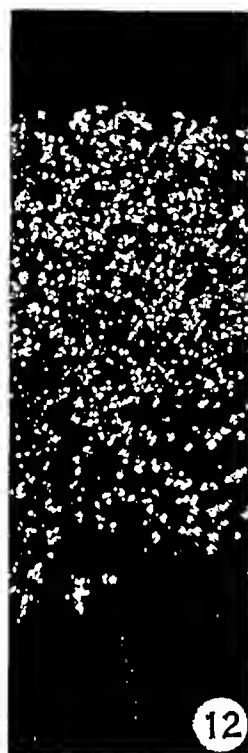


PLATE 2. LONG-TERM EXPERIMENT

much more abnormal than those of animals receiving injections of 2.5 mg. The cells appeared highly vacuolated, the sinusoids distended, and the transitional zone unusually broad.

So far as the duration of treatment was concerned, the birefringent material was already somewhat depleted at 6 hours (Fig. 10) and became progressively finer and further reduced in quantity with longer treatment. Least was detectable at 18 hours. The sudanophilic material was also somewhat depleted in the inner fasciculata at 6 hours and most at 18 hours. As before, the Schiff reaction and auto-fluorescence of any residual lipid droplets remained intense.

d. Injection of less than 10 mg hormone in periods shorter than 24 hours. In order to ascertain that the time effects noted in Exp. c were not due principally to the sizes of the doses used, groups of fasting rats were given 2.5 mg hormone on the same time schedule as above, namely, 1 injection at the 18th hour of the fast, 2 injections at 12 and 18 hours, 3 injections at 6, 12, and 18 hours, and 4 injections at 0, 6, 12 and 18 hours. The animals were killed at the end of the 24-hour fast. While the effects obtained were less marked with the small doses employed in this experiment than in Exp. c, the time relations were similar (Table 6). Adrenal steroids had already decreased at 6 hours and reached their minimum at 12 hours. Adrenal weight rose only at 18 hours. Thymus weight declined, beginning at 12 hours; liver glycogen was significantly increased only at 24 hours. The cytochemical preparations also agreed with those in Exp. c. As previously noted, the dosage of 2.5 mg caused no particular dis-

EXPLANATION OF PLATE 2

Adrenal cortices of rats tube-fed a medium-carbohydrate diet and injected with 12 mg adrenocorticotropin daily or with saline over a period of several days. All figures on this plate X75.

Figs. 12 and 13. Rats treated for 4 days.

Fig. 12. Control animal receiving saline. Birefringence preparation. Birefringent particles of mixed size are crowded in the glomerulosa and outer fasciculata, less crowded in the juxtamedullary region.

Fig. 13. Rat receiving hormone. Birefringence preparation. The cortex is broader than in the control and contains birefringent material throughout.

Figs. 14-17. Rats treated for 8 days.

Fig. 14. Control animal receiving saline. Birefringence preparation. Similar to Fig. 12.

Fig. 15. Rats receiving hormone. Birefringence preparation. Similar to Fig. 13.

Fig. 16. Control animal receiving saline. Sudan black B. Sudanophilic lipids are concentrated in the glomerulosa and outer fasciculata. No transitional zone occurs.

Fig. 17. Rat receiving hormone. Sudan black B. Lipids occur throughout the broad cortex and are unusually concentrated in the inner part of the fasciculata. No transitional zone is present. A conspicuous reticularis has developed.

Figs. 18 and 19. Rats treated for 12 days.

Fig. 18. Control animal receiving saline. Sudan IV and hematoxylin. No change has occurred in the distribution of lipids in the cortex in comparison to Fig. 16.

Fig. 19. Rat receiving hormone. Sudan IV and hematoxylin. Lipids have begun to disappear from the inner part of the cortex. Birefringent particles had likewise gone from this region. Prominent reticularis.

TABLE 6. GROSS ANATOMICAL AND CHEMICAL DATA FOR FASTED RATS RECEIVING 2.5 MG ADRENOCORTICOTROPIN 1, 2, 3, AND 4 TIMES AND KILLED 6 HOURS AFTER THE LAST INJECTION (TOTAL STARVATION, 24 HOURS).

Treatment	No. rats	Wt., g	Total liver glycogen, mg	Thymus wt., mg/100 g b. wt.	Adrenal glands wt., mg/100 g b. wt.	steroid, mg/100 mg gland
None	2	194	26	162	20.9	3.20
1 inj.	2	211	58	183	20.4	2.61
2 inj.	2	200	40	114	20.7	2.09
3 inj.	2	197	44	68	23.8	2.38
4 inj.	2	194	119	117	22.3	2.89

tortion of the adrenal cortex. However, a decline in the ketosteroid content of the zona fasciculata developed, appearing most extreme at 18 hours (Fig. 11).

These two experiments (c and d) therefore appear to corroborate the conclusions drawn from Exp. b. That is, the immediate effect of the adrenocorticotropin was to cause a release of steroid material from the cortex (6 hours). Subsequently, the adrenal enlarged, but meanwhile adrenal steroids were returning to normal levels. These results bear out the conclusions of Sayers, Sayers, Fry, White and Long (1944), who observed that steroid release preceded adrenal hypertrophy with hormone administration. Furthermore, in that paper and in later ones (Sayers, Sayers, Liang and Long, 1945 and 1946; Sayers and Sayers, 1948), these investigators have stressed the initial drop and subsequent recovery in steroid content of the adrenal under continued treatment with adrenocorticotropin. Previously, Selye (review, 1946) described the early drop and secondary recovery of sudanophilic lipid in the cortex during the "alarm" reaction to physiological stress. Most probably such observations indicate that the initial effect of adrenocorticotropin on the adrenal is to cause the immediate release of stored hormone. Thereafter the cells enlarge and begin to from ketosteroids more rapidly than usual so that, while continuing to release hormone at a high rate, they replace their cytoplasmic stores. The initial depletion, which Selye terms the "alarm" reaction, appears to take only 12 to 18 hours. Partial recovery is already apparent by the end of 24 hours.

It should be noted that significant deposition of glycogen in the liver required 18 to 24 hours, although some release of adrenal hormones had apparently already occurred at 6 hours. Such a lag between hormone release and metabolic effect suggests that a considerable period is necessary to raise the amount of carbohydrate in the blood to such a degree that glycogen deposition is increased in the liver. On the other hand, a much shorter time (6 hours) is necessary to cause lympholysis and the resulting thymus involution. This latter observation tallies with the results of Dougherty and White (1945) on mice and rabbits.

B. Long-term experiment (sample #13-10-6)

To ascertain the effects of the prolonged administration of adrenocorticotropin, adult rats were treated for up to 12 days. In this experiment, 12 mg of hormone sample #13-10-6 was administered daily (equivalent in activity to 9.8 mg of sampled, #21B used in the short-term experiments⁶). The hormone was injected in 3 equal doses of 4 mg at 8-hour intervals. Control animals received 0.5 cc of a 1 per cent solution of sodium chloride on the same time schedule. The indices of hormone effect employed were the following: thymus and adrenal weight, total adrenal steroid, and the levels of urinary nitrogen, chloride, sodium, potassium and phosphorus.

In order to obtain comparable animals for study, both the saline-injected controls and the hormone-treated rats were fed 24 cc of a medium-carbohydrate diet daily by stomach tube (Table 1). On this diet, the gain in weight of both groups proved slower than in the same animals before the institution of tube-feeding (Fig. 1) or in litter mates who ate dog chow *ad libitum* over the same period. The rats treated with adrenocorticotropin gained weight even more slowly than their controls. Also, when groups of animals were starved 24 hours before killing on the 4th, 8th and 12th days of the experiment

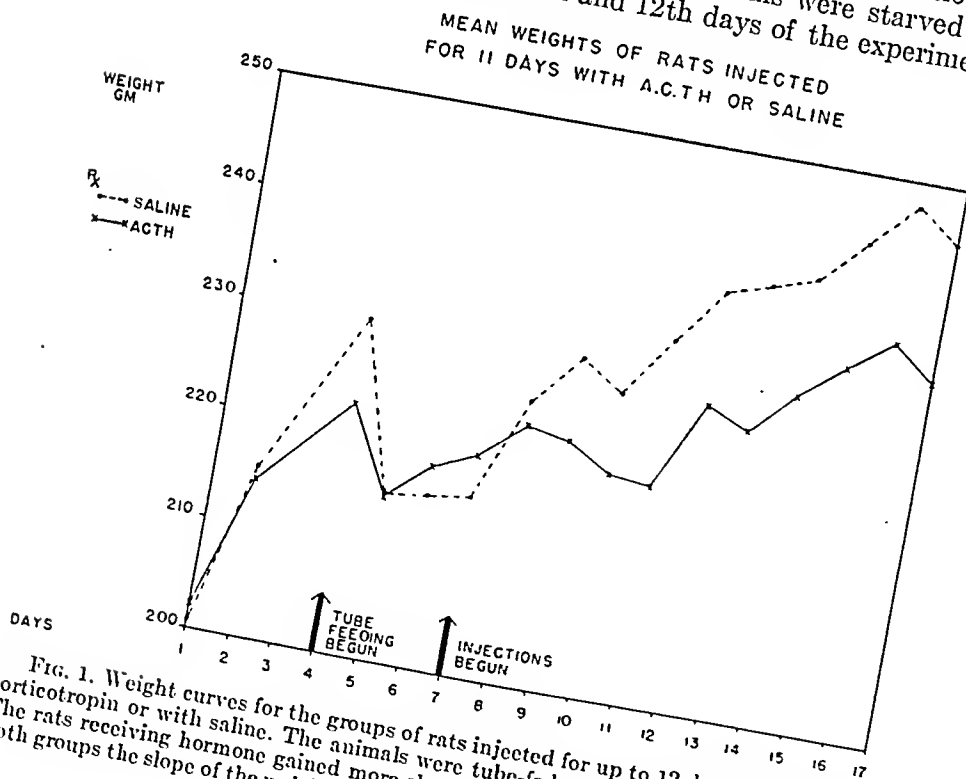


FIG. 1. Weight curves for the groups of rats injected for up to 12 days with adrenocorticotropin or with saline. The animals were tube-fed a medium-carbohydrate diet. The rats receiving hormone gained more slowly than the saline-injected controls. For both groups the slope of the weight curve was less after tube-feeding began than before.

the hormone-treated animals lost more weight than their saline-injected controls (Table 7).

Urine was collected for analysis every 48 hours during the 12 days of the experiment. The excretion of sodium, potassium and chloride was essentially the same in the hormone-treated and control rats (Fig. 2). With either treatment the excretion of all these electrolytes fell somewhat after 6 days. Also, in both the experimental and control

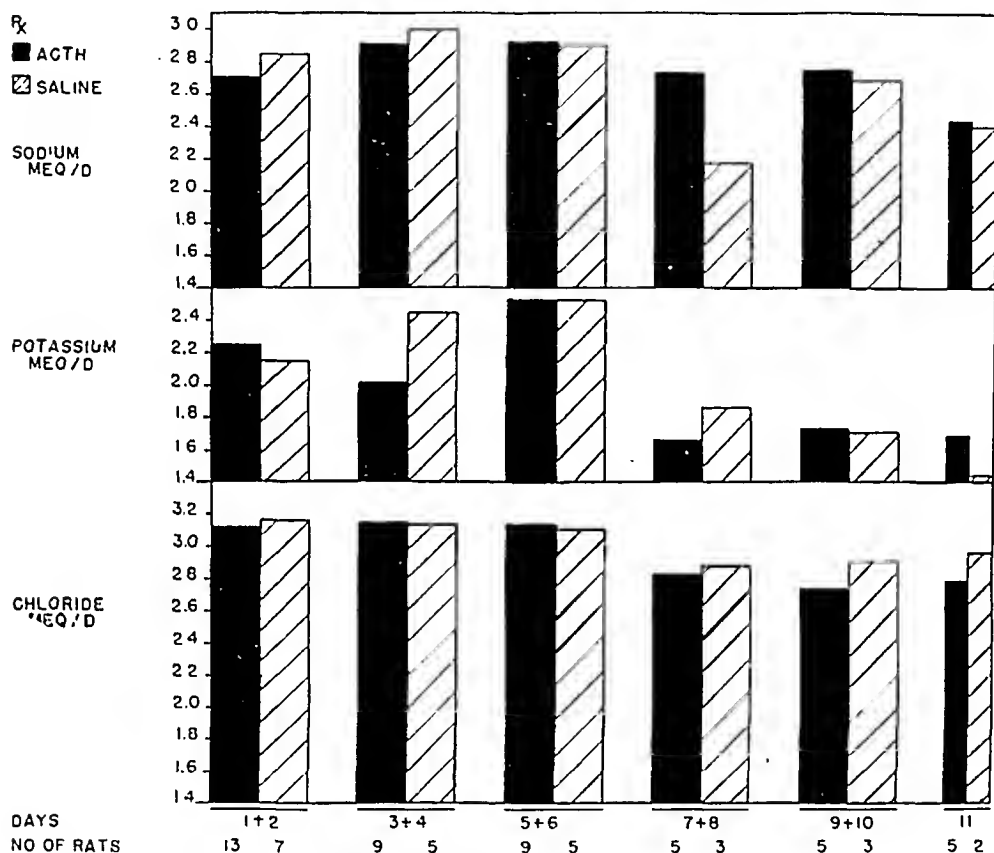


FIG. 2. Mean urinary excretion of sodium, potassium and chloride by rats injected for up to 12 days with adrenocorticotropin or with saline. Both groups excreted these electrolytes in about the same quantity.

animals, the excretion of phosphorus (phosphate) was similar and, unlike that of other electrolytes, remained constant throughout the experimental period at about 10 mg per day.

In contrast to the similar excretion of electrolytes in the hormone-treated and control animals, excretion of nitrogen was consistently greater by the rats receiving adrenocorticotropin. The experimental rats excreted 259 mg daily on the average, the controls 201 (Fig. 3). Furthermore, glycosuria occurred in the hormone-treated rats for the first 2 days of injection, but not in the controls.

Groups of hormone-injected and saline-injected rats were killed on the mornings of the 4th, 8th and 12th days, after a 24-hour fast. In

ADRENOCORTICOTROPIN ON RAT

TABLE 7. GROSS ANATOMICAL DATA FOR RATS RECEIVING 12 MG ADRENOCORTICOTROPIN DAILY IN 4 MG DOSES AT 8-HOUR INTERVALS. THE CONTROLS RECEIVED 0.5 CC 1 PER CENT SODIUM CHLORIDE ON THE SAME SCHEDULE. THE RATS WERE STARVED FOR THE 24 HOURS BEFORE NECROPSY

Treatment	No. rats	Wt., g	Wt. lost in 24 hr. starvation, g	Thymus wt., mg/100 g b. wt.	Adrenal glands wt., mg/100 g b. wt.	Adrenal glands steroid, mg/100 mg gland
Saline	2	212	4-day group			
Hormone	4	212	4.3	184		
			6.0	116	21	5.35
Saline	2	229	8-day group			
Hormone	4	210	3.8	170	29	3.55
			7.1	105	22.4	5.20
Saline	2	237	12-day group			
Hormone	5	220	3.9	155	26.4	4.78
			5.9	86	22.7	4.70
					36.5	3.26

the rats receiving adrenocorticotropin, the thymus glands were consistently smaller and the adrenal glands consistently larger than in their controls (Table 7). Moreover, total adrenal steroid was always lower in the experimental animals, although, in general, determinations for both the control and experimental animals were as high

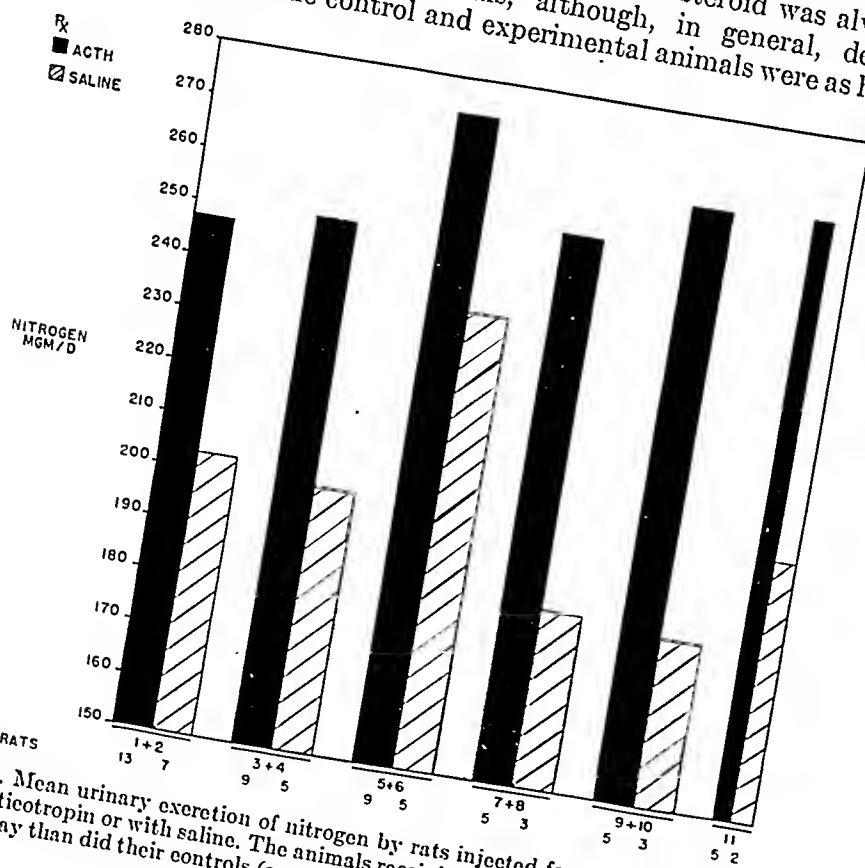


FIG. 3. Mean urinary excretion of nitrogen by rats injected for up to 12 days with adrenocorticotropin or with saline. The animals receiving hormone excreted more nitrogen each day than did their controls (average ratio, 259/201 mg per day).

or higher than the control values obtained in the short-term experiments (see Tables 2-6).

The cytochemical preparations of the adrenal glands of these animals revealed minor but consistent differences between the animals receiving hormone and those receiving saline. The cortices were invariably wider in the rats receiving hormone; this enlargement was attributable to a greater broadening of the fasciculata (Plate 2). Throughout the 12-day period, ketosteroids were abundant in the fasciculata of the control animals (Figs. 12, 14, 16 and 18). For the first 8 days, they were likewise abundant in the cortices of the rats receiving hormone (Figs. 13, 15 and 17). It is perhaps noteworthy that the distribution was more uniform in the glands of experimental animals—that is, more ketosteroid was present in the inner fasciculata than in the corresponding control glands (compare Figs. 16 and 17). By 12 days, however, lipid had almost entirely disappeared from the inner fasciculata in the hormone-treated animals and was sparser peripherally (Fig. 19). This depletion was seen in all types of preparations.

Two other observations bear comment. In all of these glands, whether from control or experimental animals, the lipid-free transitional zone between the glomerulosa and fasciculata was absent. Secondly, the cords of parenchymal cells were more conspicuously distorted in the juxtamedullary region (zona reticularis) (Figs. 17 and 19) than in normal glands of rats of this size (Fig. 4).

The chemical, morphological and cytochemical results together point to the fact that all the animals, whether receiving saline solution or hormone, experienced some adrenal stimulation. This was to be expected, since tube-feeding, mild inanition and the injections of themselves would be somewhat "alarming." Thus, the adrenals of all of the animals were larger and the thymuses smaller than normal for animals of 200 g body weight. The transitional zone had disappeared in all cases, a phenomenon which has been noted previously with physiological stress (Dalton et al., 1944). Moreover, the adrenal cortices of the control rats and of the experimental rats for the first 8 days of the experiment contained more than the normal complement of lipid droplets. Selye (1946) has termed the phase of increased lipid content in the cortex the "resistance" stage of the adaptation syndrome. That is, the adrenals had recovered from the initial depletion which characterizes the "alarm" reaction, described for the short-term experiments, and under continued stimulation, were forming more than the usual amount of hormone. More hormone was being stored than released so that an accumulation resulted.

Nevertheless, the smaller thymuses, the greater nitrogen excretion and the transitory glycosuria in the experimental rats signified a far greater release of "sugar" hormones than in the saline-injected controls. Finally, by 12 days the steroid content of the adrenals of the hormone-

treated rats had begun to decline, as evidenced both by a decrease in total steroid and by the disappearance of ketosteroid material from the inner fasciculata. Here we have indication of the approach of the so-called exhaustion stage of the adaptation syndrome—the period in which release of hormone exceeds formation and cellular stores become depleted. If this condition continued for long, the animals would die of a relative adrenal insufficiency, such as occurs in many conditions of prolonged physiological stress. The term “exhaustion” as applied to the gland itself is probably incorrect because there is no indication of a real reduction in secretory rate—the metabolic effects of the corticosteroids are maintained undiminished. The histological signs merely indicate that discharge outstrips formation, rather than that the adrenal tissue becomes fatigued.

DISCUSSION

It is well known that “normal” values for liver glycogen, total adrenal steroid, proportional organ size, etc., vary over a considerable range. As will be seen by perusing the tables presented above, variability existed from group to group in the control values. Nevertheless, within the several groups, consistent alterations from the control condition occurred with hormone treatment. Such internal consistency appears to us valid reason for presenting the data. Interpretation of the effects of this hormone, however, warrants administration of adrenocorticotropin has been given above. The increasing literature on the effects of this hormone, however, warrants a comparison of the results occurring in the rat and in man.

In both species, treatment with adrenocorticotropin appears to cause an increased secretion of hormones of the 11-oxygenated type from the cortex. In the rat this was indicated in our own experiments by the acute involution of the thymus (Dougherty and White, 1945), the increased excretion of nitrogen, the increased deposition of glycogen in the liver and the transitory glycosuria (Ingle and Thorn, 1941), as well as possibly by the temporary rise in the excretion of chloride (Thorn, Engle and Lewis, 1941). These physiological indices were accompanied by signs of increased secretory activity in the zona fasciculata. Our observations would appear to provide further substantiation of the hypothesis that the fasciculata secretes “sugar” factors and is under the control of the pituitary. They support and extend observations on the rat by Ingle and coworkers (1946, 1947). Likewise, in man, the release of “sugar” hormones appears to follow the administration of adrenocorticotropin to persons with functional adrenals (Browne, 1943; Mason et al., 1948; Forsham et al., 1948; Bartter, 1948).

On the other hand, different effects in respect to the retention of sodium chloride have been observed in man and the rat. In the rat, according to our results and those of Ingle et al. (1946, 1947), salt

retention does not follow the administration of hormone. Indeed, excretion is increased for the first day or so. Nor have we observed cytological signs of increased secretory activity by the zona glomerulosa, which probably secretes the salt-retaining hormones. These observations are compatible with earlier studies which indicated that the glomerulosa is independent of pituitary control in the rat (Deane and Greep, 1946; Greep and Deane, 1947; Deane, Shaw and Greep, 1948). In man, on the other hand, the administration of adrenocorticotropin has generally been found to cause marked salt retention following an initial period of increased excretion (Forsham et al., 1948; Conn et al., 1948; Bartter, 1948). Some of the trials on humans were performed on individuals suffering from hypopituitarism, but the same effect, though less marked, was demonstrated in normal subjects. However, Mason and coworkers (1948), using a different hormone preparation, failed to obtain this result in the one normal young woman whom they studied. All in all, there appears to be strong indication that adrenocorticotropin stimulates the release of salt-retaining hormones in man. If this is true, a species difference appears to exist in the response to adrenocorticotropin. In man the hormone stimulates the secretion of both "salt" and "sugar" hormones, whereas in the rat it induces only the release of the latter type.

Increased excretion of chloride by our rats during the first 24 hours of adrenocorticotropin administration (Table 3) may possibly have been caused, not by the increased amounts of circulating 11-oxygenated hormones, but by the posterior pituitary factors contaminating the Armour product, since control animals receiving pituitrin alone excreted more chloride than animals injected with saline (Table 2). However, Ingle and coworkers (1946, 1947) also obtained a temporary rise in sodium chloride excretion, and they were using Li's product (Li, Evans and Simpson, 1943), which is pure as tested in the Tiselius apparatus.

No rise in potassium excretion followed hormone administration in our experiments. Ingle et al. (1946, 1947) obtained a significant and consistent increase. Under the conditions of the Ingle experiments, however, the rats were actually losing weight, whereas our animals were gaining, though slowly. Since potassium is released during tissue breakdown, perhaps the difference in the proportion of protein anabolism to catabolism would account for the discrepancy between the results of Ingle and ourselves. Forsham et al. (1948) found that adrenocorticotropin induces only a transitory rise in potassium excretion in man. Further experimentation is required to elucidate this problem of potassium excretion in the two species.

SUMMARY AND CONCLUSIONS

Fasting male rats were injected with 10 mg adrenocorticotropin (Armour sample #21B) over periods of 24 hours or less. Within 6 hours

the adrenals lost considerable steroid by chemical determination, and the thymus began to atrophy. By 18 hours the adrenal glands were significantly enlarged, and by 24 hours deposition of glycogen in the liver exceeded 1000 mg per cent. The excretion of nitrogen and chloride was increased over control levels for the 24-hour period. Smaller total dosage produced similar though less marked effects.

Adult male rats were tube-fed a medium-carbohydrate diet and injected with adrenocorticotropin (Armour sample #13-10-6) at a level of 12 mg daily for a period of 12 days. They displayed hypertrophy of the adrenals, transitory glycosuria, increased nitrogen excretion and atrophy of the thymus in comparison to controls injected with saline. There were no significant differences from the controls in sodium, potassium, chloride or phosphorus excretion.

Cytochemically, none of the adrenal glands of animals treated with the hormone showed any alteration in the morphology or ketosteroid content of the zona glomerulosa. However, in the short-term experiments, the fasciculata broadened and the ketosteroid content declined, reaching a minimum at 18 hours ("alarm" reaction of Selye). The extent of the reaction depended on dosage and on the size of the animals. By 24 hours the ketosteroids had begun to be replenished. In the animals receiving hormone for many days, the fasciculata was broad; in the first 8 days the ketosteroids were increased above normal ("resistance" phase of Selye), but by 12 days they had begun to disappear from the inner part of the fasciculata ("exhaustion" phase of Selye).

These results are interpreted as indicating that adrenocorticotropin causes an increased secretion of 11-oxygenated ("sugar") hormones by the zona fasciculata of the rat but fails to increase the secretion of the salt-retaining hormones by the zona glomerulosa. After an initial release of ketosteroids in the first 18 hours, the fasciculata produces these substances faster than it releases them and consequently becomes crowded with stored ketosteroids (24 hours to 8 days). Finally, between 8 and 12 days release exceeds formation so that the zone again becomes depleted.

These results have been compared with those in man. Contrary to the observations on the rat, both types of hormone appear to be released in man following the administration of adrenocorticotropin, a result suggesting a species difference.

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THE WORK PERFORMANCE OF ADRENAL-ECTOMIZED RATS GIVEN CONTINUOUS INTRAVENOUS INFUSIONS OF GLUCOSE

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THE MUSCLE work test (Ingle, 1944) has proven to be a sensitive and reliable procedure for the bio-assay of adrenal cortex extracts and steroids which affect carbohydrate metabolism. There is indirect evidence that the early loss of muscular responsiveness in the adrenalectomized animal is due to depletion of carbohydrate in the tissues and body fluids: (1) "Fatigue" of the stimulated muscle in the adrenalectomized rat is associated with hypoglycemia; (2) There is a close parallelism between the relative potencies of adrenal cortical extracts and steroids as bioassayed by the muscle work test and by the liver glycogen deposition test (Pabst, et al., 1947); and (3) It was shown by Ingle and Lukens (1941) that the intravenous administration of glucose to the "fatigued" adrenalectomized rat gave a temporary restoration of ability to work.

The objective of the present study was to determine the effect of continuously administered glucose upon the ability of the adrenalectomized rat to continue working in response to faradic stimulation. Adrenalectomized-nephrectomized rats were used in some of the experiments because this preparation has been found to be especially sensitive to adrenal cortical hormones and is used in a bio-assay procedure (Ingle, 1944). Different loads of glucose were given by continuous intravenous infusion during 24 hours of muscle stimulation. The adrenalectomized rats tolerated greater amounts of glucose than did non-adrenalectomized rats during the time that the animals were able to work, but muscular performance was not improved by glucose except in the presence of the adrenal glands.

METHODS

Male rats of the Sprague-Dawley strain which weighed 200 ± 2 grams were used in these experiments. These animals were free from parasites and infections. The diet was Friskies Dog Cubes. The procedures used for the preparation of the animals and for the stimulation of muscle were according to Ingle (1944) with the following modifications. A Nerve Stimulator, Model B, Upjohn, was used to stimulate the gastrocnemius muscle to lift a 100-gram weight 5 times per second. The duration of each pulse was 20 milliseconds and the intensity was 15 milliamperes. The amounts of work were

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registered on automatic work recorders. Each recorder revolution represented approximately 400-gram centimeters of work.

Solutions of glucose (C. P. Dextrose, Merck) with and without beef adrenal extract (Üpjohn) were infused into the jugular vein at a constant rate by means of a continuous injection machine which delivered fluid from each of 12 syringes at the rate of 20 cc. in 24 hours. The glucose load is expressed as mg. of glucose per 100 grams of rat per hour (mg/100/h). The adrenal cortex extract (ACE) was free from alcohol and was given at the rate of 20 cc. per 24 hours. This amount was selected because it has been estimated (Ingle, Li and Evans, 1946) that the adrenal cortices of an intact rat can secrete the activity equivalent of at least 20 cc. of ACE under stimulation of adrenocorticotrophic hormone.

Analyses of glucose were made on tail blood by the method of Miller and Van Slyke (1936).

EXPERIMENTS AND RESULTS

Experiment 1 was a study of the glucose tolerance and work performance of nephrectomized rats and adrenalectomized-nephrectomized rats. The following glucose loads were studied in this experiment: 20, 40, 85, 125, 150, 175, and 200/100/h. The following experimental conditions with 6 rats each were represented at each glucose load: adrenalectomized-nephrectomized without treatment; adrenalectomized-nephrectomized given saline only; adrenalectomized-nephrectomized given glucose only; adrenalectomized-nephrectomized given glucose in 20 cc. of adrenal cortex extract; adrenalectomized-nephrectomized given adrenal cortex extract in saline; nephrectomized given saline; and nephrectomized given glucose. The muscle was stimulated until it ceased to respond, or for 24 hours. Analyses were made for blood glucose at the end of 6 hours of stimulation and again at the end of 24 hours in those rats which survived. Figure 1 shows the rate of work and the level of blood glucose at the end of 6 hours in relationship to the experimental conditions and the glucose load. Figure 2 shows the total amount of work for each group and the level of blood glucose and rate of work at the end of 24 hours in those animals which survived. In each figure the data from the animals not given glucose which served as controls at each glucose load are averaged together according to the experimental condition represented.

The intravenous administration of glucose to adrenalectomized-nephrectomized rats without ACE did not improve the work performance above the very low level shown by the untreated animals. This was true even when the glucose loads were sufficiently large to prevent a fall in blood glucose. Similarly there was no clearly established tendency for the administration of glucose to improve the performance of the ACE-treated animals. At glucose loads of 175 and 200/100/h the performance of the ACE-treated rats was poor. The performance of the animals having intact adrenal glands was increased in proportion to the glucose load. Within a period of 6 hours the adre-

nalectomized-nephrectomized rats without ACE showed significantly lower levels of blood glucose at each glucose load than did the animals having the adrenal glands intact. The values for blood glucose were increased by the administration of ACE but did not rise to the levels shown by the non-adrenalectomized animals at comparable glucose loads even during 24 hours.

Experiment 2 was a study of the glucose tolerance and work performance of sham-operated and adrenalectomized rats. Glucose loads

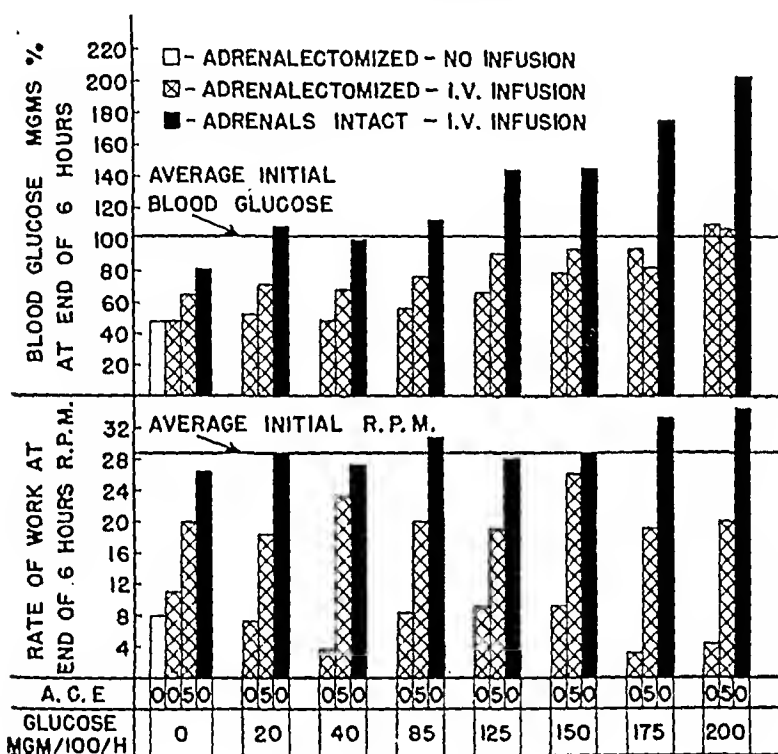


FIG. 1. Nephrectomized rats. Effect of adrenalectomy upon work and blood glucose at the end of 6 hours as related to glucose load. Each average for groups given glucose represents 6 rats. Each average for pooled groups not given glucose represents 35 rats.

of 100, 150, and 200/100/h were used. The following experimental conditions with 6 rats each were represented at each glucose load: adrenalectomized without infusion; adrenalectomized given saline; and adrenalectomized given glucose in saline. Eleven sham-operated rats were run in parallel with the adrenalectomized animals. Stimulation was continued until the muscle ceased to respond. Some of the adrenalectomized rats "fatigued" within 24 hours and all collapsed and died within 48 hours. In contrast, the sham-operated rats continued to work for periods of 120 to 168 hours. Although hypoglycemia was prevented by glucose loads of 150 and 200/100/h, the work per-

TABLE 1. TOTAL AMOUNTS OF WORK (RECORDER REVOLUTIONS) PERFORMED BY ADRENALECTOMIZED AND SHAM-OPERATED RATS. AVERAGES AND RANGE

Experimental Condition & Treatment	Number of Rats	Total Work
sham-operated	11	152181
saline only		(111863-180262)
adrenalectomized no infusion	18	20617
adrenalectomized saline only		(11785-38383)
adrenalectomized glucose (100/100/h)*	18	23798
adrenalectomized glucose (150/100/h)*		(15136-37902)
adrenalectomized glucose (200/100/h)*	6	26425
		(17586-35237)
	6	16750
		(13728-21712)
	6	13022
		(10699-16360)

* Glucose loads of 100 and 150/100/h failed to prevent a fall in blood glucose during the period that the animals continued to work but with a glucose load of 200/100/h the average level of blood glucose was 140 mg. per cent at 6 hours and 367 at 24 hours.

formance of these groups was depressed below the performance of rats treated with 100/100/h or with saline only. The rats having intact adrenal glands performed several times as much work as the adrenalectomized animals. The data on total work are summarized in Table 1.

Experiment 3 was a study of the effect of glucose in water and of

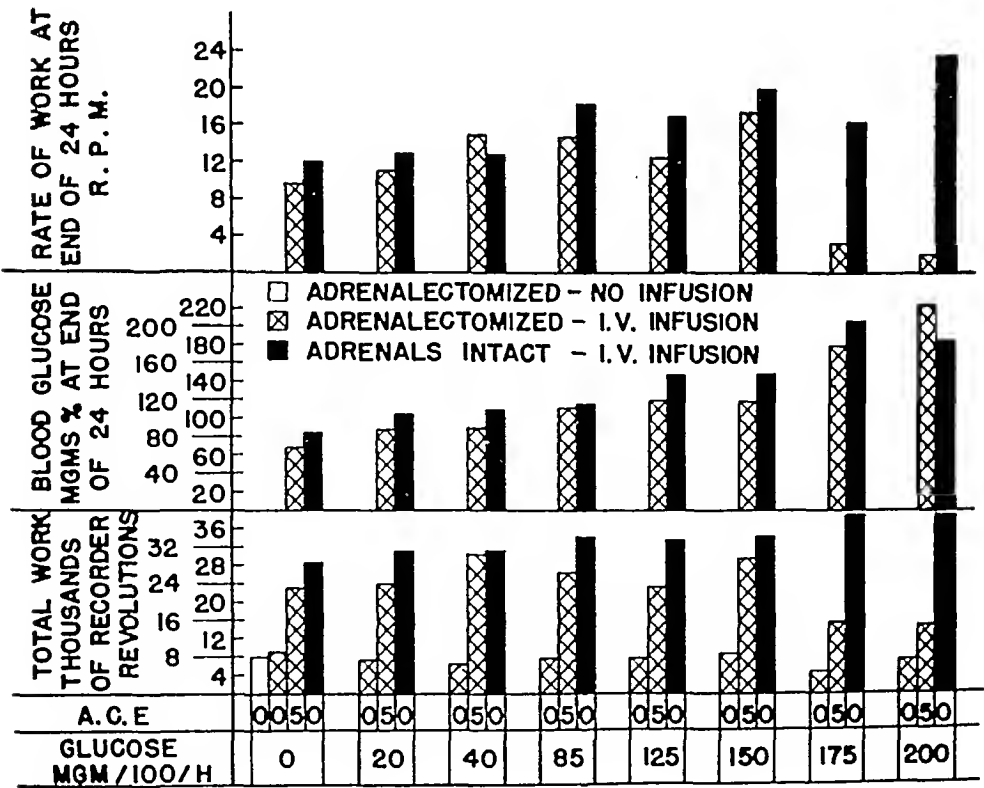


FIG. 2. As in Figure 1 but at end of 24 hours.

glucose in saline upon the work performance of adrenalectomized-nephrectomized rats. Glucose loads of 100, 200 and 250/100/h were used. Six rats were represented in each experimental group. There was a tendency for those animals which received intravenous infusions of glucose in saline to work better than rats which received glucose in water but the differences were small and the work performance of all of the animals remained very poor as compared to the performance of similar rats treated with ACE or having intact adrenal glands. The data are summarized in Table 2.

In Experiment 4 the levels of blood glucose and work performance were noted at 2-hour intervals in 6 pairs of adrenalectomized-nephrec-

TABLE 2. A COMPARISON OF THE EFFECT OF INFUSIONS OF GLUCOSE IN WATER AND GLUCOSE IN SALINE UPON THE BLOOD GLUCOSE AND WORK PERFORMANCE OF ADRENALECTOMIZED-NEPHRECTOMIZED RATS.* AVERAGES AND RANGE OF INDIVIDUAL VALUES. SIX RATS PER GROUP

Glucose load mg./100/h	6 hour blood glucose mg. per cent		6 hour work rate rev. per min.		Total work recorder revolutions	
	Water	Saline	Water	Saline	Water	Saline
100	50 (39-59)	59 (50-71)	12 (8-25)	22 (16-28)	9786 (7853-13017)	14001 (11296-16232)
200	123 (71-254)	121 (71-200)	7 (2-12)	11 (8-20)	8758 (7805-9916)	9940 (7502-11427)
250	127 (69-251)	109 (72-227)	4 (0-20)	4 (0-12)	8550 (6776-12421)	9545 (7519-12578)

* Each average is based upon 6 animals.

tomized rats and nephrectomized rats. Fourteen hours was the longest time that all of the adrenalectomized-nephrectomized rats lived. At a glucose load of 100/100/h the blood glucose of the non-adrenalectomized rats increased above the initial values and remained elevated. The rate of work was well sustained. In contrast, the blood glucose of the adrenalectomized-nephrectomized animals decreased rapidly to hypoglycemic levels and remained low until death. The rate of work also declined rapidly and by 8 hours all of the adrenalectomized-nephrectomized rats were in collapse. These data are summarized in Figure 3.

Six pairs of adrenalectomized-nephrectomized and nephrectomized rats were given a glucose load of 200/100/h for 14 hours. The blood glucose of the non-adrenalectomized animals increased rapidly to hyperglycemic levels during the first 4 hours and then showed some decrease. The rate of work was well sustained in these animals. The blood glucose of the adrenalectomized rats showed a smaller initial rise than that of their controls, followed by a fall to normal values, but by the 12th and 14th hours the blood glucose had increased to values much higher than in the non-adrenalectomized series. The rate

of work decreased rapidly so that muscular responsiveness was lost in all of the adrenalectomized animals by 8 hours. These data are summarized in Figure 4.

In Experiment 5 the levels of blood glucose and work performance were determined at 4-hour intervals in adrenalectomized and in sham-operated rats. Twenty hours was the longest period of time that all of the adrenalectomized rats survived. Six pairs of rats were given a glucose load of 100/100/h. The blood glucose of the non-adrenalecto-

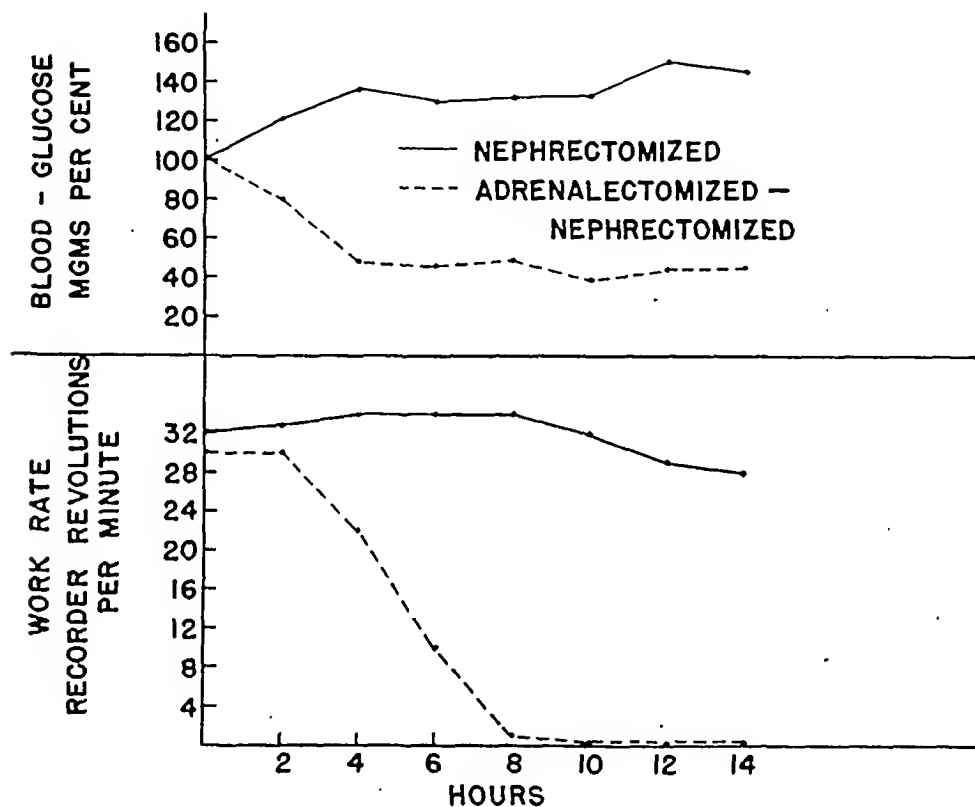


FIG. 3. Work performance and blood glucose level in nephrectomized and adrenalectomized-nephrectomized rats given a glucose load of 100/100/h. Six rats per group.

mized rats increased above the initial values and remained elevated. The rate of work decreased only a little during 20 hours. The adrenalectomized rats showed some fall in blood glucose during the first 12 hours and thereafter a rise which almost reached the level of the non-adrenalectomized animals at 20 hours. The work output of the adrenalectomized rats fell to a low level by 12 hours. These data are shown in Figure 5.

Six pairs of adrenalectomized and sham-operated rats were given a glucose load of 200/100/h for 20 hours. The non-adrenalectomized animals developed a mild hyperglycemia and the rate of work fell only a little during 20 hours. The blood glucose of the adrenalectomized animals showed a smaller initial rise than their controls but by the 16th and 20th hour the blood glucose increased to much higher

values than in the sham-operated controls. The rate of work fell rapidly and by 12 hours all of the animals were either collapsed or near collapse. These data are summarized in Figure 6.

DISCUSSION

The data of these experiments show that the continuous intravenous administration of glucose to the adrenalectomized rat does not

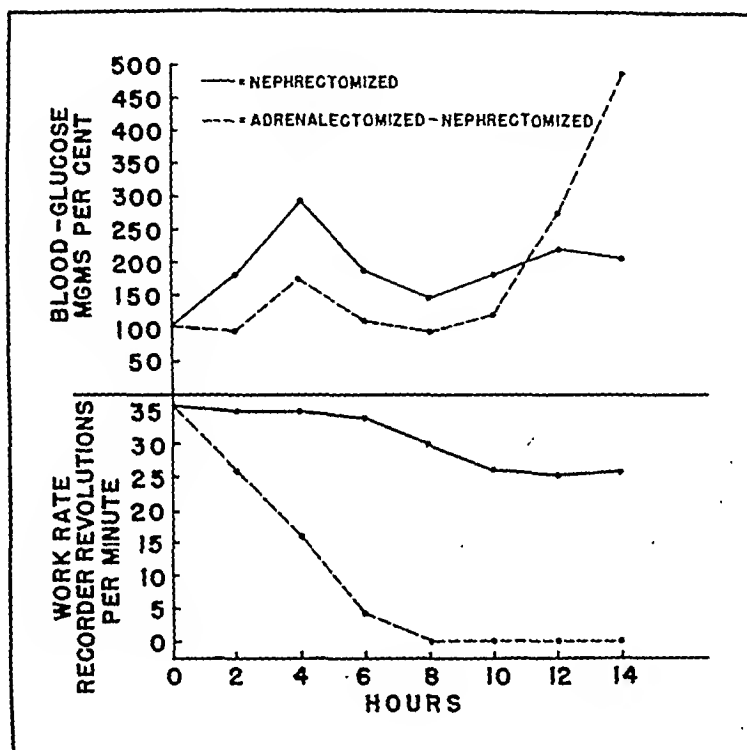


Fig. 4. Work performance and blood glucose level in nephrectomized and adrenalectomized-nephrectomized rats given a glucose load of 200/100/h. Six rats per group.

significantly improve work performance. This result was not anticipated since Ingle and Lukens (1941) found that a single intravenous injection of a solution of glucose in the "fatigued" adrenalectomized rat gave a temporary improvement in rate of work which was significantly greater than was obtained with osmotically equivalent solutions of sucrose and of sodium chloride. We have consistently confirmed this finding of Ingle and Lukens. It is possible that the hypoglycemia of the "fatigued" adrenalectomized rat is a critical factor in limiting work output but when hypoglycemia is prevented, failures of other physiological systems continue to limit muscular responsiveness.

Kendall *et al.* (1938) infused adrenalectomized dogs with solutions

of glucose at the rate of 100/100/h for 3 hours. These animals tolerated the glucose as well as normal dogs but developed anuria when glucose alone was given. When either adrenal cortex extract or 0.9 per cent sodium chloride was added to the solution of glucose a mild diuresis developed. When the injections were stopped the adrenalectomized dogs without treatment developed weakness and prostration. It was concluded that sodium chloride was the full equivalent of adrenal

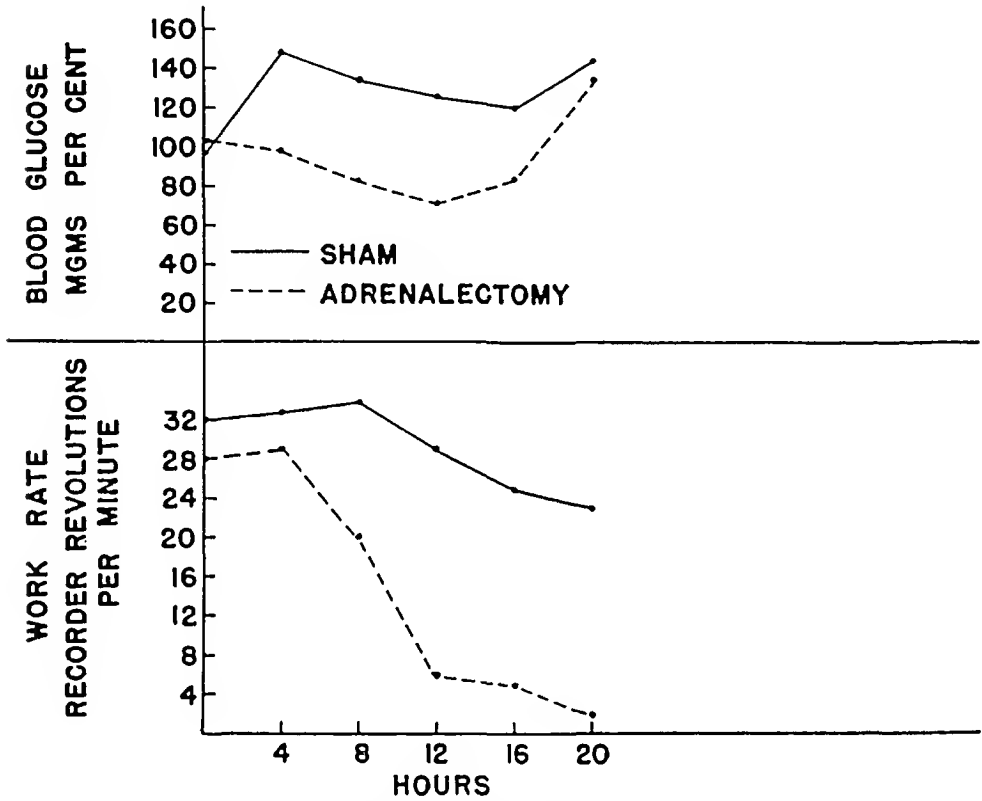


FIG. 5. Work performance and blood glucose level in sham-operated and adrenalectomized rats given a glucose load of 100/100/h. Six rats per group.

cortex extract for maintaining normal resistance to the injection of glucose. In the present study the addition of 0.9 per cent sodium chloride to the solution of glucose improved the work performance to a very small extent (Table 2).

During the time that the adrenalectomized rats continued to work, their tolerance for intravenously administered glucose was greater than that of adrenalectomized animals treated with ACE or of animals having their adrenal glands intact. These data suggest that there was no impairment of the ability of the adrenalectomized rats to utilize glucose during the time that the animal continued to work. A full appraisal of the extent of utilization would require a determination of the fate of the exogenously administered glucose, of the extent of gluconeogenesis and of shifts in carbohydrate stores. We do not have this information.

The results of Experiments 4 and 5 (Figures 3, 4, 5 and 6) illustrate two dimensions of metabolic problems: (1) The time-response relationship, and (2) The load-response relationship. The adrenalectomized animal not given glucose or given a small load of glucose generally manifest hypoglycemia at any time after the onset of adrenal cortical insufficiency and up to the time of death. In Experiment 4 the adrenalectomized-nephrectomized rats given a glucose load of 100/100/h developed hypoglycemia which continued until death

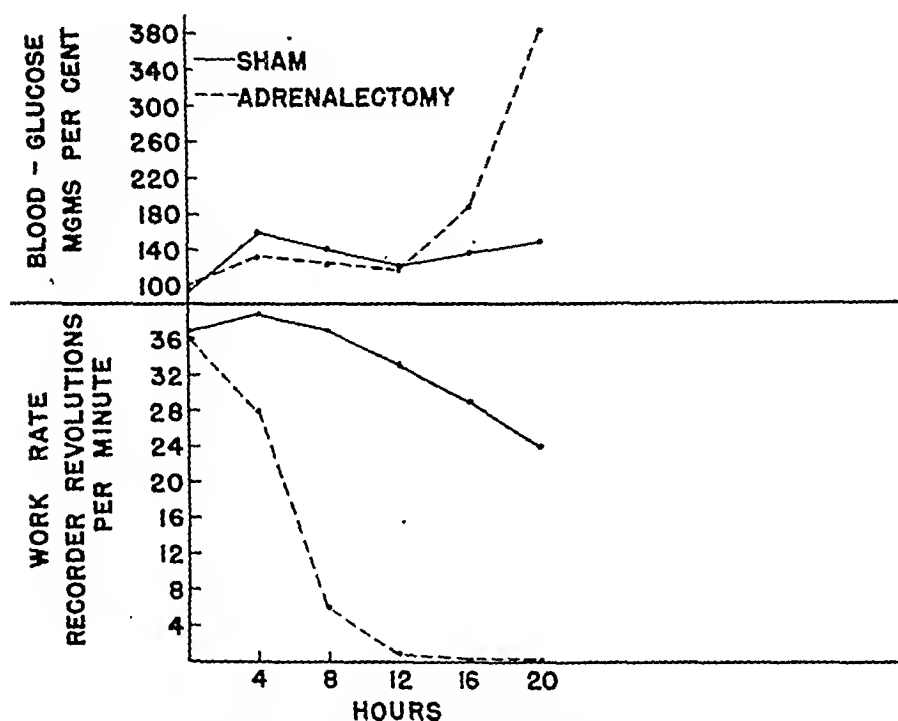


FIG. 6. Work performance and blood glucose level in sham-operated and adrenalectomized rats given a glucose load of 200/100. Six rats per group.

while the non-adrenalectomized-nephrectomized rats showed a rise in blood glucose. When the load was increased to 200/100/h the adrenalectomized animals showed lower values for blood glucose during the time that they continued to work but as muscular responsiveness was lost and as the animals approached death the level of blood glucose rose sharply above that of the non-adrenalectomized animals. Similar findings were noted in Experiment 5. Ingle and Nezamis (1948) have made related observations on adrenalectomized and non-adrenalectomized eviscerated rats given continuous intravenous infusions of glucose for 24 hours. During the early hours after operation the blood glucose values were lower in the adrenalectomized series at each glucose load, and at the end of 24 hours the adrenalectomized animals which received small glucose loads had lower values

for blood glucose but at high glucose loads the order of tolerance was reversed.

There is evidence that the adrenal cortical hormones inhibit the utilization of carbohydrate and that the adrenally insufficient animal utilizes increased amounts of carbohydrate. How can hormones which seem to inhibit the utilization of carbohydrate sustain an optimal ability to work? It may be possible to rationalize the relationship as follows: The C-11, oxygenated steroids of the adrenal cortex may spare the oxidation of carbohydrate under basal conditions or at moderate rates of energy output. These hormones may sustain or stimulate the ability of the animal to mobilize protein and fat for energy purposes. The proneness of the adrenally insufficient animal to develop hypoglycemia may be due in part to an inability to mobilize and utilize fat and protein for energy purposes to a normal extent. Physiological amounts of the cortical hormones probably do not limit the ability of the animal to attain a peak of carbohydrate oxidation at high and continued levels of energy output.

The administration of adrenal cortex extract intravenously at the rate of 20 cc. in 24 hours did not sustain the ability to work as well as intact adrenal glands. In our experience we have never been able to sustain a completely normal ability to work by means of adrenal cortex extracts or steroids. It is possible that the absence of the adrenal medulla is of physiological importance not only from the standpoint of work performance but of glucose tolerance.

If the fatigability of the adrenalectomized rat is not due to lack of available carbohydrate and if there is no inability to assimilate glucose from the blood during the time that the muscle is failing, what is the basis for loss of muscular responsiveness in the adrenally insufficient animal? The early studies of Hales, Haslerud and Ingle (1935) led to the conclusion that the "fatigued" adrenalectomized rat under these conditions is in circulatory collapse. Ingle and Lukens (1941) also recognized circulatory failure as a factor in "fatigue." Our observations indicate that the collapse represents low blood pressure and a greatly reduced blood flow. It seems probable that there is some failure in energy exchange basic to this type of circulatory collapse (Swingle and Remington, 1944). No investigator has probed deeply into these problems. The findings of the present study do rule out the very simple explanation for "fatigue" as due solely to lack of glucose.

SUMMARY

A study was made of the effect of continuous intravenous infusions of glucose upon the ability of adrenalectomized rats to respond to the faradic stimulation of the gastrocnemius muscle. Different loads of glucose were administered to nephrectomized and to adrenalectomized-nephrectomized rats with and without adrenal cortex extract (ACE). Glucose without ACE did not improve work performance

above the very low level shown by untreated animals even when hypoglycemia was prevented. Similar results were obtained on adrenalectomized rats having the kidneys intact. During the time that adrenalectomized or adrenalectomized-nephrectomized rats continued to work the blood glucose values remained lower than for non-adrenalectomized rats at each glucose load. At a high glucose load (200/100/h) the adrenally insufficient animals showed lower blood glucose values until muscular responsiveness was lost, whereupon the blood glucose curve rose rapidly to values much higher than were shown by the non-adrenalectomized controls.

Although exhaustion of available carbohydrate may be one factor limiting the work performance of the adrenally insufficient rat, the prevention of hypoglycemia by the intravenous administration of glucose does not sustain the responsiveness of stimulated muscle in these animals.

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ASSOCIATION NOTICE

POSTGRADUATE COURSE IN ENDOCRINOLOGY

A postgraduate course in Endocrinology, sponsored by the Association for the Study of Internal Secretions, will be held at the Skirvin Hotel in Oklahoma City, February 21-26, 1949.

The faculty will consist of outstanding clinical and research endocrinologists of the United States and Canada. The program will consist of clinics and demonstrations and will be a practical one of equal interest to those in general medicine and the specialists.

The fee will be \$100 for the entire course and applications will be accepted in the order received. Applications should be directed to Henry H. Turner, M. D., Secretary-Treasurer, 1200 North Walker, Oklahoma City, Oklahoma.

ASSOCIATION AWARDS FOR 1949

THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb & Sons Award of \$1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner; 1941—Dr. Philip E. Smith; 1942—Dr. Fred C. Koch; 1943—no award was given; 1944—Dr. E. A. Doisy; 1945—Dr. E. C. Kendall; 1946—Dr. Carl G. Hartman; 1947—Drs. Carl F. and Gerty T. Cori; 1948—Dr. Fuller Albright. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russel; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl G. Heller. The work cited may be either in the field of preclinical or clinical endocrinology. The Award is for \$1,200.00. If within 24 months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, the Award will be increased to \$1,800.00.

THE AYERST, McKENNA & HARRISON FELLOWSHIP

The Ayerst, McKenna & Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin, and in 1948 to Dr. Ernest M. Brown, Jr. This Fellowship is designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. The Fellowship may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend for the Fellowship will vary in accordance with the qualifications of the appointee, but will not exceed

ASSOCIATION AWARDS

October, 1948

\$2,500.00 The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. The nominee must present evidence or scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals; submit a program of proposed study; indicate one or more institutions where the proposed program shall be carried out; submit statements of approval from the investigators with whom he proposes to conduct his research.

Each member has the privilege of making one nomination for each award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to endocrinology and by a bibliography of the nominee's most important publications, and reprints, if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 15, 1949.

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GONADOTROPHIC HORMONES AND OVARIAN HYPEREMIA IN THE RAT

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THE OVARIAN hyperemia reaction has been employed as a definitive endpoint in a rapid biological test for the early detection of pregnancy. This particular response of the ovary to urinary gonadotrophins was first noticed by Eberson and Silverberg in 1931 and is characterized by a congestion of the vessels of the ovarian tissue resulting in macroscopic reddening of the ovaries. The vasodilatation occurs soon after the administration of chorionic gonadotrophin and has been observed as early as one hour after intraperitoneal injection of urine of pregnancy (Kupperman, 1948). The hyperemic reaction while first described in the ovaries of immature albino rats has also been noted within six hours after the administration of pregnancy urine in the corpora lutea of ovaries of adult rats. A similar reaction was also observed in the ovaries of adult mice and immature hamsters (Kupperman et al., 1943; Kupperman and Greenblatt 1946-1948). The hyperemic effect noted in hamsters and adult mice was not consistently observed until 15 hours after subcutaneous or intraperitoneal administration of the urine. The reaction failed to develop or was not observed macroscopically in the ovaries of immature albino mice (Rockland strain) or immature or mature guinea pigs.

While the ovarian hyperemia response of the immature rat ovary to urinary gonadotrophins is generally accepted and has been employed with varying degrees of success as a test for the early diagnosis

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of pregnancy (Kelso, 1940; Frank and Berman, 1941; Salmon et al., 1942; Kupperman et al., 1943, 1948; Zondek, Sulman and Black, 1945; Riley et al., 1948) there is no agreement as to the factor or principle in the urinary gonadotrophins responsible for the production of ovarian hyperemia. In preliminary studies it was noted that this reaction occurred only after the administration of urine or extracts rich in luteinizing or luteotrophic hormones (Kupperman & Greenblatt, 1946). Extracts containing chiefly follicle stimulating hormone failed to induce ovarian hyperemia. These observations were substantiated by Zondek, Sulman and Black (1945) who believed that ovarian hyperemia is evoked mainly by the luteinizing hormone and that the follicle stimulating hormone has only an augmentative effect. On the other hand, Farris (1946) has reported ovarian hyperemia occurring after the administration of follicle stimulating hormone. In view of these dynamically opposite results, an extensive study was planned to note the role of various fractions of the gonadotrophic complex in the production of ovarian hyperemia. The following report is concerned with the effect of fractionated and unfractionated gonad stimulating extracts prepared from the pituitary gland, urine and serum on the induction of ovarian hyperemia in the immature female rat.

METHODS AND MATERIALS

Preparation of extracts: The follicle stimulating preparations used in this study were prepared by the tryptic-digestion method of McShan and Meyer (1940). The crude sheep pituitary gonadotrophic fractions (ISAP and Vetrophin-Abbott) and the cow pituitary fraction (IBAP140A) are the pH 5 soluble fractions that were recovered by precipitation with acetone and dried by washing with acetone or by lyophilizing according to the method reported by McShan and Meyer (1943). The luteinizing preparations (RLH and PLH) are the pH 5 and pH 4 insoluble fractions, respectively, and the purified gonadotrophic preparations (F) were obtained by methods reported by the same authors (McShan and Meyer, 1943). The lactogenic preparations (Lac and LacR) were made by the method of Bates and Riddle (1935). The purified horse gonadotrophin was obtained from E. R. Squibb & Sons, Incorporated, and the horse pituitary powder (AP17) was made by dehydrating the fresh glands with acetone. The rat pituitary gonadotrophin (RAP126) was obtained by aqueous extraction of air-dried (Kupperman, Elder and Meyer, 1941) rat pituitary glands. This extract was dried by lyophilizing. The purified pregnant mare serum gonadotrophin (PMS-E1P3) was prepared by the method of Cartland and Nelson (1937). The crude prolan preparations (ETOH and CPU105HM2) were obtained by alcohol precipitation of the chorionic gonadotrophin from human pregnancy urine. The thyrotrophic extract was prepared by the Armour Laboratories.¹

Assay for ovarian hyperemia effect (six hours): The extracts to be tested were administered by the subcutaneous instead of the intraperitoneal route to remove the possibility that the solvents employed in the preparation of the extracts would cause irritative hyperemia by direct contact and thereby

¹ Supplied through the courtesy of Dr. F. F. Fenger.

produce a false positive reaction. While the extracts to be tested were administered in various doses, a constant amount of 2 cc. of aqueous diluent was always employed. The animals were sacrificed by ether asphyxiation six hours after administration of the hormone preparation. Twenty-five to 35 day-old female rats of a Wistar strain were employed. The viscera were exposed without trauma, and with no loss of blood. The ovaries were then examined for evidence of hyperemia and were classified as positive (those showing definite congestion), doubtful (ovaries exhibiting some slight vasodilation), and negative (no evidence of stimulation). The ovaries were permitted to remain exposed 2-3 minutes after autopsy to allow for a maximum expression of the hyperemic reaction.

Assay for gonad stimulating action (4.5 days): The extracts employed were assayed for their gonadotrophic effect by the administration of a given amount of the extract in 9 divided aliquots injected in 0.5 cc. portions once on the afternoon of the first day and twice daily for the next four days with autopsy performed on the morning of the 6th day. The extracts were administered in the above manner to 21 day-old intact female rats of the Sprague-Dawley strain. Qualitative and quantitative responses of the ovaries were noted.

While comparison between results obtained with the 4.5 day gonadotrophic assay and the six-hour hyperemic method could not be made on a quantitative basis, the comparative activity of the different preparations was established by each of the two assay methods. In addition the relative effectiveness of the various extracts in inducing ovarian hyperemia was compared on the basis of milligram equivalent of dried pituitary powder to the gonad stimulating effects of the same preparation.

RESULTS AND DISCUSSION

The data in Table 1 compare the gonad stimulating effect of different follicle stimulating hormone (FSH) preparations with the ovarian hyperemia inducing action of the same preparations. The extracts were all assayed in intact, non-hypophysectomized rats and proved to be principally follicle stimulating in action. Further assay of one preparation (FSH130) in 1 and 2.5 gram doses in immature male rats (Table 2) indicate that this extract exhibited no growth stimulating effect upon the prostate and seminal vesicles while increase in testicular weight was attained. These findings are in agreement with the prevalent concept of the dual action of FSH and luteinizing hormone (LH) on the seminiferous tubules and interstitial cells, respectively, and attest to the high degree of purification attained in the preparation of the FSH extract.

Analysis of the data presented in Table 1 indicate that at least 6-8 times the dose of FSH necessary to induce follicular stimulation associated with a 3-4 fold increase in ovarian weight is necessary before ovarian hyperemia may be produced. In addition findings are presented in Table 1 to show that FSH preparations less pure than others produce doubtful hyperemic responses in the ovaries in doses of 2 grams (FSH312 and FSH523). These findings imply that while ovarian hyperemia is not an expression of the FSH activity it may be

induced when sufficient LH is present in the administered preparation.

The effect of fractionated (other than FSH) and non-fractionated pituitary, urinary and serum extracts upon the induction of ovarian

TABLE 1. THE COMPARATIVE GONAD STIMULATING AND OVARIAN HYPEREMIA EFFECT OF FOLLICLE STIMULATING HORMONE (FSH) EXTRACTS

Preparation	Gonad Stimulating Action				Ovarian Hyperemia		
	Dose*	Number of Rats	Ovarian Weight	Ovarian Response	Dose*	Number of Rats	Reaction
FSH 130	0.5 gm.	3	43	All Follicles	1 gm.	2	Negative
	1.0 gm.	3	89	All Follicles	2 gm.	3	Negative
					3 gm.	2	Doubtful
					4 gm.	2	Positive
FSH 129	0.5 gm.	9	53	All Follicles	2 gm.	1	Negative
	1.0 gm.	3	85	Few C. L.**	3 gm.	1	Negative
FSH 312	0.5 gm.	3	94	Cloudy Follicles	1 gm.	2	Negative
					2 gm.	3	Doubtful-Negative
					3 gm.	1	Positive
FSH 313	0.5 gm.	6	61	All Follicles	2 gm.	1	Negative
					4 gm.	1	Positive
FSH 508	0.5 gm.	6	58	All Follicles	2 gm.	1	Negative
FSH 509	0.5 gm.	6	58	All Follicles	1 gm.	1	Negative
					2 gm.	1	Negative
FSH 522	0.5 gm.	7	42	All Follicles	2 gm.	1	Negative
					4 gm.	1	Negative
FSH 523	0.5 gm.	9	74	Few C. L.** in 3 rats All Follicles, otherwise	2 gm.	1	Doubtful
					3 gm.	1	Positive
FSH 5256	0.5 gm.	6	28	All Follicles	2 gm.	1	Negative
	.75 gm.	9	48	All Follicles	3 gm.	1	Negative
	1.0 gm.	3	71	All Follicles			

* In grams equivalent of dried pituitary powder.

** A total 1-7 corpora lutea in both ovaries of each rat.

TABLE 2. THE EFFECT OF FSH 130 ON THE TESTES AND MALE ACCESSORIES OF THE IMMATURE RAT

Dose ¹ (gram)	No. of Rats	Testes (mgm)	Seminal ² Vesicles (mgm)	Prostate (mgm)
0	3	506	9.5	37.0
1.0	3	655	9.3	33.5
2.5	3	676	9.5	37.8

¹ Equivalent of dried pituitary powder.

² Less the coagulating gland.

hyperemia is presented in Table 3. These data indicate that in addition to the ovarian-hyperemic stimulating effect of unfractionated gonadotrophic extracts, highly purified LH and lactogenic (luteotrophic) extracts—in contrast to the FSH preparations—are also effective agents in producing ovarian hyperemia. The unfractionated aqueous extracts of both sheep and beef pituitary glands produce ovarian hyperemia in doses of 25 and 125 mg. equivalent, respectively. Quantitative assay of the beef pituitary extract over a period to 4.5

TABLE 3. GONAD STIMULATING AND OVARIAN HYPEREMIA EFFECT OF UNFRACTIONATED AND FRACTIONATED GONADOTROPHIC, LACTOGENIC AND THYROTROPHIC PREPARATIONS

Kind of gonadotrophic preparation	Gonad Stimulating Action			Ovarian Response**	Ovarian Hyperemia		
	Total Dose*	Number of Rats	Ovarian Weight (mgm)		Dose*	Number of Rats	Hyperemia
Crude unfractionated: Beef IBAP140A	500 mg. (+ 6 mg. Heme)	3	106	Several-Numerous Corpora Lutea	50 mg.	2	Negative
					125 mg.	1	Positive
					500 mg.	1	Positive
Sheep ISAP32A	100 mg.	9	52	Follicle-Numerous C. L.	10 mg.	3	Negative
					25 mg.	2	Positive
ISAP24	100 mg.	6	94	Numerous C.L.	50 mg.	1	Positive
					25 mg.	2	Positive
					50 mg.	2	Positive
Vetrophin (Abbott)	100 mg.	6	93	Few C. L.	100 mg.	2	Positive
					10 mg.	3	Negative
					25 mg.	1	Positive
Horse AP17	15 mg.	3	97	Follicular Stimulation	50 mg.	2	Positive
					10 mg.	2	Negative
Rat RAP126	1 mg.	10	58	Several C. L.	5 mg.	3	Negative
	2 mg.	3	95	Several C. L.	10 mg.	2	Negative
	3 mg.	2	145	Several C. L.	0.5 mg.	2	Negative
					1.0 mg.	3	Negative
					2.0 mg.	2	Negative
Purified unfractionated: Sheep F115A	100 mg.	3	71	Few-Several C. L.	3.0 mg.	2	Negative
					4.0 mg.	2	Negative
F116A	100 mg.	12	84	Follicles-Several C. L.	10 mg.	3	Negative
					25 mg.	1	Positive
Hog F119A	100 mg.	6	39	Follicular Stimulation	50 mg.	3	Negative
					100 mg.	2	Negative
Horse Gonadotrophin (Squibb)	2 units	3	21	Cloudy Follicles	1 unit	3	Negative
	5 units	3	84	Numerous C. L.	2 units	3	Positive
	10 units	3	261	Many C. L.	5 units	2	Positive
	15 units	3	337	Many C. L.	15 units	1	Positive
Pregnant Mare Serum PMSE1P3	0.25 mg.	3	85	Numerous C. L.	0.05 mg.	2	Negative
					0.1 mg.	4	Positive
					0.25 mg.	3	Positive
Chorionic PETOH	3 cc.	3	43	Few C. L.	3 cc.	2	Positive
CPU105	4 cc.	3	53	Few-Several C. L.	4 cc.	2	Positive
Fractionated sheep: luteinizing hormone (LH) P115	500 mg.	3	16	Negative	50 mg.	2	Negative
					100 mg.	3	Positive
					250 mg.	1	Positive
R28A	500 mg.	1	15	Negative	100 mg.	2	Negative
					250 mg.	2	Positive
P116BC	500 mg.	2	13	Negative	25 mg.	3	Negative
					50 mg.	2	Positive
					100 mg.	2	Positive
					250 mg.	1	Positive
LH15	500 mg.	2	17	Negative	25 mg.	2	Negative
					50 mg.	4	Positive
					75 mg.	2	Positive
Lactogenic hormone LacR124	500 mg.	3	10	Negative	50 mg.	2	Negative
					100 mg.	2	Positive
LacR900	500 mg.	3	12	Negative	500 mg.	2	Positive
Lac52	500 mg.	2	10	Negative	500 mg.	2	Positive
					1000 mg.	2	Positive
Thyrotrophic hormone	5 units	3	13	Negative	5 units	2	Negative
	10 units	3	14	Negative	10 units	2	Negative

* Mg. refers to mg. equivalent of dried pituitary powder.

** Few C. L. = A total of 1-7 corpora lutea in both ovaries

Several C. L. = A total of 8-14 corpora lutea in both ovaries

Numerous C. L. = Too many corpora lutea to count

Many C. L. = Ovary composed principally of corpora lutea

days according to the method described above in doses equivalent to 0.5 grams of dried powder failed to produce any detectable ovarian stimulation. However, as little as 125 mg. equivalent induced ovarian hyperemia. Incorporation of 6 mg. of heme with 0.5 gram equivalent of beef pituitary extract resulted in ovarian stimulation when the material was administered over a period of 4.5 days (McShan and Meyer, 1937). Luteinizing hormone extracts (P118, R28A, P116BC, LH15) caused ovarian hyperemia in doses as small as 100 mg. equivalent of dried pituitary glands. The LH preparations are 20-40 times as effective in the production of ovarian hyperemia as FSH when compared on the basis of equivalents of dried pituitary glands. These same luteinizing extracts were ineffective in exhibiting gonad stimulating action when administered over a 4.5 day period to the immature 21 day-old female rat. However, when the LH preparations were administered in conjunction with FSH extracts, corpora lutea were produced. Lactogenic preparations were effective in producing ovarian hyperemia in doses which failed to show evidence of gonad stimulating activity (Table 3). One preparation (LacR124) induced ovarian hyperemia in doses equivalent to 100 mgm. of dried pituitary powder and compares favorably with the similar activity of LH preparations.

Pregnant mares' serum, human pregnancy urine extracts and commercially available unfractionated sheep pituitary preparations all possessed LH activity and readily produced ovarian hyperemia. Administration of a thyrotrophic extract and suspension of dried horse pituitary failed to induce hyperemia of the ovarian tissue. The thyrotrophic preparation exhibited no gonad stimulating action in the immature rat and the crude horse pituitary extract showed only follicular stimulating activity in the doses employed. Hence it may be concluded that the absence of sufficient LH in these preparations prevented the induction of ovarian hyperemia.

Our results have consistently demonstrated that extracts possessing LH or luteotrophic activity are capable of producing ovarian hyperemia. In addition it may be noted that while highly purified FSH extracts are practically ineffective in producing ovarian hyperemia, FSH enhances the ovarian hyperemic action of LH. This is supported by data in Table 3 where it is shown that unfractionated gonadotrophic extracts (ISAP24) are as a rule more effective in producing ovarian hyperemia than LH preparations when a comparison between the minimal hyperemic stimulating dose (on basis of milligram equivalents of dried pituitary powder) is made. The augmentative effect of FSH in the presence of LH upon ovarian hyperemia was also demonstrated by Zondek et al. (1945). The ovarian hyperemic stimulating action of lactogenic hormone may be ascribed to its luteotrophic properties since it has been shown that lactogenic preparations will maintain the function of the corpus luteum (Astwood, 1941; Evans,

Simpson and Turpeinen, 1938). On the other hand, gonad stimulating extracts which are predominantly follicle stimulating in action fail to induce ovarian hyperemia except when excessive doses of these preparations are employed. There is, however, one exception that we have noticed to the above concept that LH preparations will produce ovarian hyperemia, namely it was observed that when a gonadotrophic extract prepared from rat pituitary glands was administered to immature female rats ovarian hyperemia failed to develop. Ovarian hyperemia was not observed despite the fact that doses far exceeding the minimal dose necessary to produce ovarian hypertrophy and corpora lutea formation (4.5 day assay) were employed. Although no explanation is at hand to account for the failure of rat pituitary extracts to induce ovarian hyperemia in the rat, it is possible that homologous pituitary preparations are not hyperemic in action notwithstanding their LH activity.

The hyperemic inducing effects of LH and luteotrophic extracts suggest the use of this end-point as a rapid assay method for establishing luteinizing and luteotrophic activity of unqualified gonadotrophic preparations. Such a procedure may serve as a means of rapidly screening a large number of compounds in testing for such activity. The ovarian hyperemia response may also be employed in quantitative assays for LH or luteotrophic hormone in the serum of normal and pregnant women and in those patients presenting suspected pathological findings such as chorionepithelioma and hydatidiform mole when greatly increased levels of urinary gonadotrophins are present. In addition it has recently been suggested (Zondek et al., 1948) that diagnosis of fetal death may be made by noting quantitatively the ovarian hyperemia stimulating action of various dilutions of human pregnancy urine. The greatest dilution at which ovarian hyperemia is attained may be used as the end-point for quantitative assays. Thus the hyperemia unit (OHU) may be defined as the least amount of material or greatest dilution of serum or urine which will produce ovarian hyperemia in all of the animals injected six hours after subcutaneous administration of the preparation to immature female rats, 25-30 days of age and 45-70 grams in weight.

While our earlier results (Kupperman and Greenblatt, 1946) and the present observations are in agreement with the findings of Zondek, Sulman and Black (1945), we are at a loss to explain the discrepancies between our findings and those of Farris (1946). This latter worker has ascribed the hyperemic response of the rat ovary to gonadotrophins as due to the FSH activity of the preparations. While we have employed the same end-point, different methods of asphyxiation of the test animal have been used. Whereas ether has been the lethal agent in our experiments, Farris has adopted the use of illuminating gas. Perhaps the use of such an agent could sufficiently alter the appearance of the ovaries to explain the difference.

SUMMARY

Ovarian hyperemia has been employed as a definitive end-point in the rapid (2-6 hours) pregnancy test utilizing the rat as the test animal. Studies were undertaken to ascertain the gonadotrophic factor responsible for evoking ovarian hyperemia. The following preparations from pituitary glands, serum and urine were tested: Unfractionated pituitary extracts and fractionated pituitary preparations i.e. follicle stimulating hormone, luteinizing hormone, lactogenic and thyrotrophic extracts; pregnant mare serum, and pregnancy urine extracts. Some pituitary preparations were administered as aqueous suspensions of dried pituitary powder. Only those preparations exhibiting luteinizing or luteotrophic activity were capable of inducing ovarian hyperemia when administered according to the described procedure. A comparison between the hyperemic action and the gonadotrophic potency of the various extracts was made. In view of the specificity of the luteinizing and the luteotrophic hormones in evoking ovarian hyperemia it has been suggested that this end-point be employed as a rapid procedure in screening gonadotrophic compounds for their luteinizing and luteotrophic effects. An ovarian hyperemic unit is described and its use in an assay method is discussed.

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THE CRITICAL REQUIREMENT FOR PANTOTHENIC ACID BY THE ADRENALECTOMIZED RAT¹

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INTRODUCTION

IN EARLIER experiments it was observed that survival in adrenalectomized rats was significantly prolonged when the diet was supplemented with very large doses of pantothenic acid and the rats were allowed 1% NaCl as drinking water (Ralli, 1946). In view of this finding we were interested in determining the critical dose of pantothenic acid necessary for the prolonged survival of rats following adrenalectomy. This report is concerned with the effect of daily doses of pantothenic acid varying from 0.03 to 6 mg. on survival in adrenalectomized rats.

EXPERIMENTAL

Black rats of the Long-Evans strain, bred in the laboratory, were placed on a diet deficient in pantothenic acid (Ralli and Graef, 1943) when 30 days of age. This diet contained 22% vitamin-free casein, 64% sucrose, 9% Primex, and 5% of the modified salt mixture² containing no NaCl. 100 gm. of the basal diet was supplemented with 1.9 cc. cod liver oil, 0.3 mg. thiamine hydrochloride, 0.3 mg. pyridoxine hydrochloride, and 0.9 mg. riboflavin. The deficient diet was continued for 30 days, by which time the failure of the rats to gain weight, graying of the fur, and the small excretion of pantothenate in the urine³ all indicated that depletion of pantothenic acid had occurred. The rats were then adrenalectomized. Following adrenalectomy the diet was supplemented daily with the dose of calcium pantothenate to be tested. These dosages will be presented as the amount of pantothenic acid equivalent to the dose of calcium pantothenate given. In order to avoid unnecessary manipulations of the animals, and also to distribute the dose of pantothenic acid throughout the day, 0.5 ml. of a solution of calcium pantothenate (made up to contain 2 daily doses

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² The composition of the modified salt mixture was 2 gm. ferric citrate, 38 gm. calcium diphosphate, 36 gm. potassium citrate, 10 gm. magnesium citrate, 0.04 gm. copper sulphate, and 0.005 gm. potassium iodide.

³ We are indebted to Dr. Saul H. Rubin, Director of the Nutrition Laboratories, Hoffmann-LaRoche, Inc., Nutley, New Jersey, for the pantothenate assays of rat urine.

per ml.) was mixed with the amount of food each rat was expected to eat during the next 24 hours. Food consumption records were kept for all animals and the total quantity of food offered was adjusted to each rat's appetite. Throughout the survival experiments all rats received a 1% solution of NaCl as drinking water. The rats were weighed at frequent intervals.

Some rats in each group were deprived of salt at intervals throughout the experiment in order to test the completeness of adrenalectomy. Other animals which had survived adrenalectomy for long periods were subjected to stress in order to evaluate their condition. These rats were obliged to swim for 25 minutes in water at 25° C. Blood sugar was determined in tail blood before and after swimming by the method of Polis and Sortwell (1946).

RESULTS

Table 1 shows the effects on survival after adrenalectomy of varying doses of pantothenic acid daily. In all experiments in which the

TABLE 1. EFFECT OF PANTOTHENIC ACID ON SURVIVAL
IN ADRENALECTOMIZED RATS

NaCl	Pan. acid daily mgm.	No. of rats	Per cent of total surviving						Mean S.D.
			10	25	50	75	100	150	
			Days						
1% in water	0	105	50	2	0	0	0	0	11.9±5.4
	0.03	19	74	0	0	0	0	0	14.2±4.3
	1.0	15	87	7	0	0	0	0	
	2.0	14	93	43	36	29	29*		
	3.0	31	74	16	16	16	16*		
	3.5	14	71	43	43*				
	4.0	22	96	77	73	50	41*		
	4-6	70	100	91	81	67	62	50	
None	0	26	7	0					6.2±2.5
	4-6	21	62	0					12.5±4.5

* Experiments discontinued . . . animals used for special studies.

rats received up to 4 mg. of pantothenic acid daily, the calcium pantothenate was administered to each rat in the amounts indicated in the table. The animals indicated as receiving 4 to 6 mg. of pantothenic acid daily were supplied *ad lib.* with a diet containing 4 mg. of calcium pantothenate in 10 gm. of diet. The amount of diet consumed daily was weighed during periods of the experiment and, although the daily intake of the diet varied, most rats ingested an average of about 5 mg. of pantothenic acid per day (Ralli, 1946). The control group consisted of adrenalectomized rats which were continued on the deficient diet after adrenalectomy.

The necessity for NaCl has previously been reported (Ralli, 1946). Without salt, the rats survived only a few days after adrenalectomy, whether or not they were receiving pantothenic acid. The survival for the rats without salt is also given in the table.

The results show that survival following adrenalectomy was sharply curtailed in animals continued on the deficient diet and 1% NaCl. When 0.03 mg. of pantothenic acid daily was fed, the mean survival was slightly longer, but survival was still strictly limited. This amount of pantothenic acid has been shown to be more than

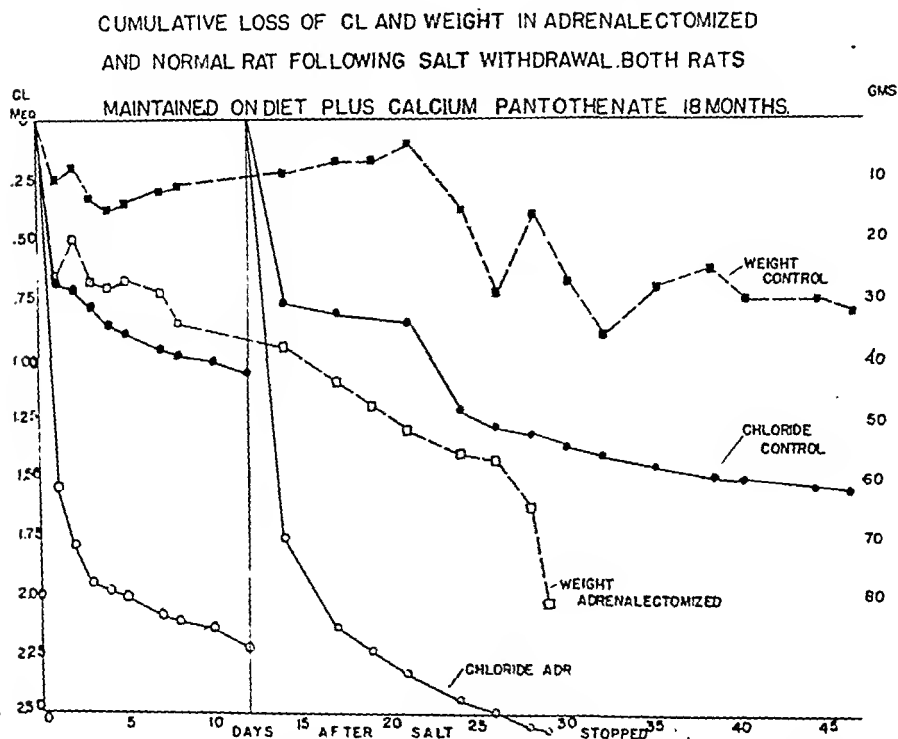


FIG. 1. Effect of salt withdrawal in an adrenalectomized and a normal rat. The 2 rats were placed on a diet deficient in pantothenic acid when 36 days of age. After 30 days on the diet one rat was adrenalectomized and the other rat was sham operated, and in both rats the diet was supplemented with calcium pantothenate (4 to 6 mg.) and a 1% NaCl solution. After 18 months NaCl was stopped. At this time the adrenalectomized rat weighed 272 gms, and the control animal weighed 207 gms. The adrenalectomized rat survived salt withdrawal for 28 days and during this time it lost 51 gms. in body weight. The control rat during the same period of time lost 22 gms. The control rat continued to survive and was finally sacrificed after 6 weeks. The line at 12 days indicates a break in the experiment when through an error NaCl was administered for a short period of time.

sufficient for optimal growth by intact rats 10 weeks of age (Unna and Richards, 1942). On 1 mg. of pantothenic acid daily a larger percentage of the animals survived for 10 days, and one animal (7%) survived for 25 days. The completeness of adrenalectomy in this animal was checked by withdrawing salt and the animal succumbed within 26 hours.

When the dose of pantothenic acid was increased to 2 mg. or more

daily, there was a definite increase in percentage survival, and in some of each group prolonged survival was obtained. On 2, 3, and 3.5 mg. daily less than half of the rats survived for 25 days or more. When the daily dose of pantothenic acid was increased to 4 mgm., 77% of the rats lived for 25 days or more. When the rats ingested the diet *ad lib.* and the daily intake of pantothenic acid varied from 4 to 6 mgm., 91% of the animals survived adrenalectomy for more than 25 days.

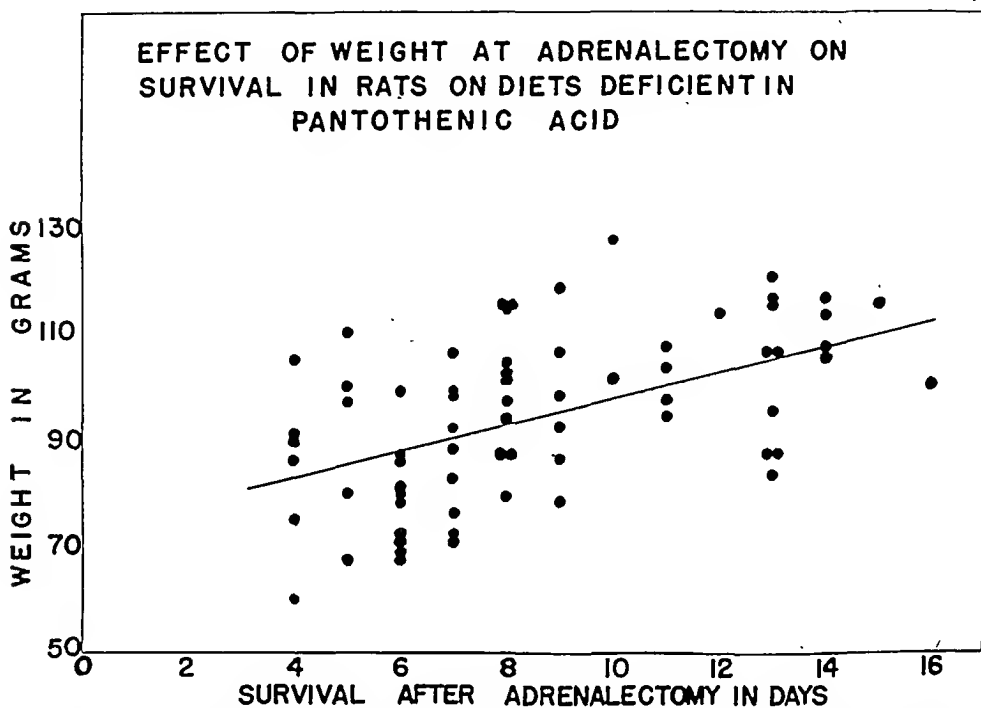


FIG. 2. The weight in grams at the time of adrenalectomy is plotted against the survival after adrenalectomy in days. All rats had been maintained on a diet deficient in pantothenic acid for 30 days before adrenalectomy and were continued on the same diet plus 1% NaCl after adrenalectomy.

The completeness of adrenalectomy was checked in many of the long-surviving animals by depriving them of NaCl. All of the rats tested succumbed to salt deprivation, usually within 2 to 4 days. An occasional rat which had been maintained on NaCl and pantothenic acid for a long time following adrenalectomy withstood NaCl deprivation for a much longer period. While the completeness of adrenalectomy might be questioned in these cases, both the weight and chloride losses were much greater in the adrenalectomized rats than in their intact controls on the same regime. Figure 1 shows the cumulative loss of weight in grams and of chloride in milliequivalents of such an adrenalectomized rat compared with its intact control after salt was withheld from both. The adrenalectomized rat had survived adrenalectomy for 18 months at the time salt was withdrawn. This animal survived adrenalectomy for an unusually long period and also sur-

vived salt withdrawal for a total of 28 days. The survival following salt withdrawal was influenced by a break in the experiment when through an error salt solution was given to both rats, and this, as indicated in the chart, occurred on the 12th day. Withdrawal of salt again was associated with a sharp drop in weight in the adrenalectomized rat and in a negative chloride balance. In the intact control there was a loss of weight, but this became stabilized after the animal had lost approximately a total of 30 gm. A similar loss of chloride and weight was reported (Ralli, 1946) in other animals surviving adrenalectomy for prolonged periods.

Analysis of the data on 105 control animals which were continued on the deficient diet following adrenalectomy showed that the weight

TABLE 2. THE SURVIVAL INDEX* OF ADRENALECTOMIZED RATS

Pantothenic acid mg./day	Number of rats	Mean weight at adr. gms.	Median survival after adr. days	Survival* index
0.0	10	95	8	1.1
0.0	15	102	8	0.9
0.0	11	80	6	0.9
0.03	19	120	12	1.0
1.0	15	83	13	1.9
2.0	14	80	20	2.9
3.0	31	85	15	2.1
3.5	14	98	18	2.2
4.0	22	100	76	9.5
4-6	70	103	150	16.7

* The survival index is the ratio of the median survival of rats on a given intake of pantothenic acid to the expected survival of rats of the same weight (± 10 gms.) on a diet deficient in pantothenic acid.

of the rat at the time of adrenalectomy is one factor affecting its survival following the operation. This data is shown in Figure 2 in which the weight of the rat is plotted against its survival after adrenalectomy. The regression line was calculated by the method of least squares. This same tendency for the heavier animals to survive longer after adrenalectomy than the lighter ones could be demonstrated in all groups of rats except those receiving 4 to 6 mg. of pantothenic acid daily.

Since weight is one factor influencing survival after adrenalectomy, we wished to express the survival of rats receiving various doses of pantothenic acid in a way which allowed for this variable. We have, therefore, devised a survival index based on the mean weight of the animals in each group at adrenalectomy. The survival index is the ratio of the median survival of rats on a given intake of pantothenic acid to the expected median survival of rats of the same weight, ± 10 grams, on a diet deficient in pantothenic acid. Table 2 shows the survival index for each group of adrenalectomized rats with reference to the dose of pantothenic acid received. As a partial check on the

validity of this procedure, the survival index has been calculated separately for each of 3 small groups of control animals chosen at random. All give a result close to the theoretical value of 1.0. Rats receiving 0.03 mg. of pantothenic acid daily also showed a survival index of 1, indicating that the slight extension of survival on 0.03 mg. shown in Table 1 was probably due to the fact that this group of

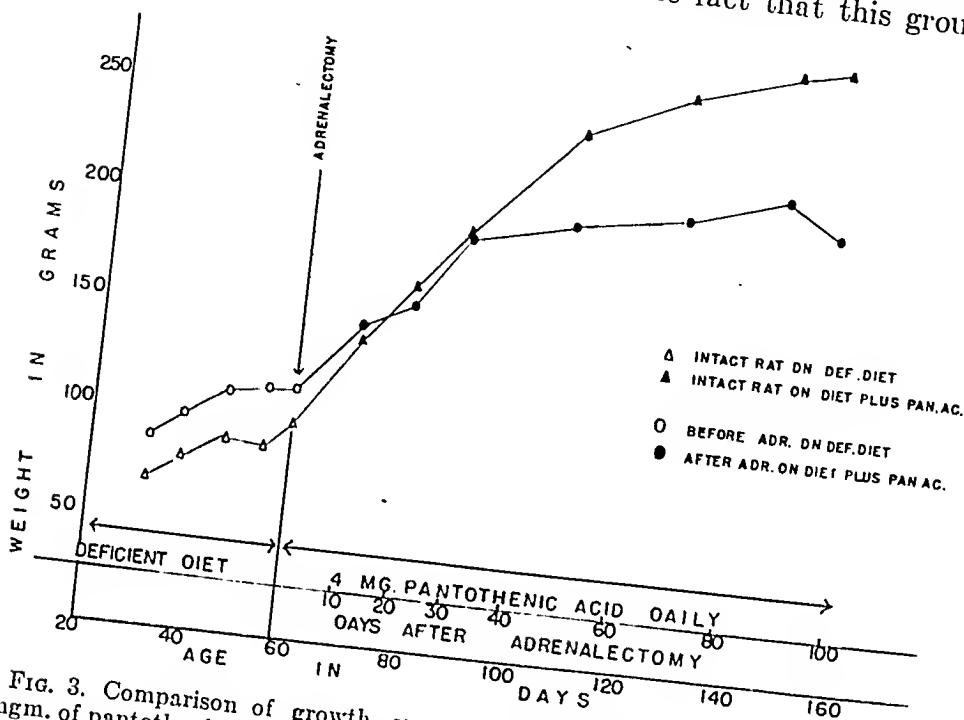


FIG. 3. Comparison of growth curves in adrenalectomized and intact rats on 4 mgm. of pantothenic acid daily. Average growth curves of 4 intact and 4 adrenalectomized male rats. All rats were placed on a pantothenic acid deficient diet when 32 days of age and were continued on the diet for 26 days. At this time 4 of the rats were adrenalectomized and 4 were continued as intact controls and each rat received 4 mgm. of pantothenic acid daily.

animals was unusually heavy. Intakes of 1, 2, 3 and 3.5 mg. of pantothenic acid daily approximately doubled the expected median survival, and therefore doubled the survival index, giving values ranging from 1.9 to 2.9. On 4 mg. of pantothenic acid daily the survival index increased strikingly to a value of 9.5 and on 4 to 6 mg. the survival index was 16.7. In other words, while a few individual rats may survive adrenalectomy for prolonged periods on diets providing up to 3.5 mg. of pantothenic acid daily, 4 mg. or more of pantothenic acid are required for any marked extension of survival in more than half the animals.

When adrenalectomized rats were fed large doses of calcium pantothenate they not only survived but appeared well and grew almost normally for a time. The maximum weight attained was, however, consistently less than that of intact rats on the same diet. In Figure 3

average growth curves are shown for 4 intact male rats and for 4 adrenalectomized male rats. All the animals were placed on the pantothenic-acid deficient diet when 32 days of age. Half the animals were adrenalectomized at 58 days of age and all received 4 mg. of pantothenic acid daily from 58 days until the conclusion of the experiment. Each point represents the mean weight of 4 rats at the indicated age.

For about a month after adrenalectomy the intact and adrenalectomized rats kept pace with each other fairly closely. The temporary interruption in the rate of growth which occurred in the adrenalectomized rats between 10 and 20 days after adrenalectomy is characteristic

TABLE 3. EFFECT OF SWIMMING ON BLOOD SUGAR

Condition of rat	No. of rats	Diet or supplement	Initial blood sugar mg. %	Final blood sugar mg. %	% Change in blood sugar
Intact	4	Nuchow	134 \pm 7	112 \pm 11	-16 \pm 9
2 Days after Adrenalectomy	3	Nuchow	133 \pm 10	93 \pm 12	-31 \pm 12
6 Days after Adrenalectomy	4	Nuchow	138 \pm 11	95 \pm 9	-32 \pm 6
Intact Rat on Exp. Diet for 100 Days	3	4 mg. Pan. per day	125 \pm 9	133 \pm 20	+ 6 \pm 13
Adr. Rats 100 Days after Adr. on Exp. Diet	3	4 mg. Pan. per day	136 \pm 7	122 \pm 5	-11 \pm 2

and has occurred consistently. It seems to reflect a critical period in the adjustment of the adrenalectomized rat to the loss of its cortical hormones. Animals on an inadequate intake of pantothenic acid were likely to die during this period, and those which survived usually continued to survive for relatively long periods.

About 30 days after adrenalectomy the rate of gain of the adrenalectomized animals dropped below that of the intact animals. The maximum weight of the adrenalectomized rats was reached about 80 days after adrenalectomy, when the rats were 140 days of age. At this time the average weight of the adrenalectomized rats was 220 grams, while the intact rats weighed an average of 275 gm. and were still gaining.

Similar curves have been constructed using data from female rats. The relationship between the weights of adrenalectomized and intact rats was similar to that found in males, although the absolute weights were lower.

It should be emphasized that these adrenalectomized rats receiving adequate amounts of pantothenic acid and NaCl appeared well and, as will be shown below, were capable of muscular work.

Since the ability of an animal to withstand stress is considered a significant indication of adrenal cortical function, we have subjected adrenalectomized and intact rats to swimming as a form of stress. The rats were placed in large stone crocks containing water at 25° C and were obliged to swim for 25 minutes. Tail blood was taken for the determination of blood sugar before and immediately after swimming. The ability of a given animal to withstand stress was judged by the effect of the swim on the animal's blood sugar.

The data are summarized in Table 3, where the initial and final blood sugar values are shown for intact and adrenalectomized rats on Nuchow, and for intact and adrenalectomized rats on the experimental diet providing 4 mg. of pantothenic acid daily. The values shown are the means and standard deviations of the means.

The data show that the initial blood sugar values were essentially the same for all groups of animals tested, but that their ability to maintain this value after swimming varied widely. The intact animals receiving 4 mg. of pantothenic acid daily showed a slight, though probably insignificant, rise in blood sugar following swimming. The adrenalectomized rats on the same diet showed a moderate decrease of 11%, which was less than the 16% loss in the intact Nuchow rats and very much less than the 30% loss in adrenalectomized rats on Nuchow.

DISCUSSION

These observations point to the importance of pantothenic acid in the diet of adrenalectomized rats. The amount required is so large in comparison with the dietary needs of intact rats that it seems unlikely that its action is merely one of overcoming a deficiency in pantothenic acid.

The adrenalectomized rats on large doses of pantothenic acid were in good physical condition. They appeared well and could not be easily distinguished from the intact animals. The fur in both the intact and adrenalectomized rats was thick and well pigmented. The adrenalectomized animals were somewhat less active than the intact animals and their food intake was slightly less. For a time there was little difference in weight between the 2 groups but ultimately the intact rats outstripped the adrenalectomized rats. By the 100th postoperative day there was a considerable difference in the average weight of the 2 groups.

The stress experiments showed that these adrenalectomized animals were in unusually good condition for rats 100 days after adrenalectomy which had received no hormone therapy at any time. Pantothenic acid prevented the fall in blood sugar which would have been expected in adrenalectomized rats subjected to stress and which was observed in the adrenalectomized rats on Nuchow. One reason for the prolonged survival of these animals may be that large doses of pantothenic acid protect rats from the disturbance in carbohydrate

metabolism which ordinarily follows adrenalectomy. It has been postulated repeatedly (Williams, 1941; Wright, 1942; Berkman, Dorfman and Koser, 1942; Hills, 1943) that pantothenic acid is related in some way to the carbohydrate metabolism of normal animals, possibly through participation in a coenzyme system (Lipmann et al., 1947; Naehmansohn and John, 1945). The effectiveness of pantothenic acid in maintaining life in the adrenalectomized rat may be associated with its action on carbohydrate metabolism.

Another way in which pantothenic acid may be contributing to survival in the adrenalectomized animal is in maintaining water balance. Gaunt and his associates (1946) showed that pantothenic-acid deficient rats were very sensitive to large doses of water and that water intoxication was prevented by the administration of either pantothenic acid or adrenal cortical extract. The combined administration of NaCl and pantothenic acid to adrenalectomized rats may have compensated in some way for the loss of the adrenal hormones in respect to water and salt metabolism.

The physiological disorder caused by adrenalectomy is not confined to a single function, and pantothenic acid without NaCl is ineffective—but NaCl is equally ineffective in maintaining survival of adrenalectomized rats on diets deficient in pantothenic acid. Our experiments did not define the role of pantothenic acid in the adrenalectomized rat, but the amount of pantothenic acid necessary for survival is now established and its importance is again demonstrated.

SUMMARY

Black rats 30 days of age were placed on a diet deficient in pantothenic acid. After 30 days on the deficient diet the animals were adrenalectomized. The adrenalectomized rats received the same diet plus graded amounts of pantothenic acid ranging from 0.03 mg. to 6 mg. daily. Throughout the experiments, the rats were given 1% NaCl as drinking water.

Prolonged survival following adrenalectomy was observed in more than half the rats receiving 4 mg. or more of pantothenic acid daily. A few animals receiving from 1 to 3 mg. of pantothenic acid survived adrenalectomy for prolonged periods.

The weight of the rat at the time of adrenalectomy was shown to be one factor influencing the subsequent survival of animals receiving 3.5 mg. or less of pantothenic acid.

Adrenalectomized rats receiving 4 mg. or more of pantothenic acid daily grew normally for about 4 weeks postoperatively. Thereafter the intact rats outstripped the adrenalectomized animals.

Rats 100 days after adrenalectomy, maintained on 4 mg. of pantothenic acid daily, showed less decrease in blood sugar following swimming than either intact or 6-day adrenalectomized rats on Nuchow.

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A SIMPLIFIED TECHNIQUE FOR HYPOPHYSECTOMY OF THE DOMESTIC FOWL

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THE SURVIVAL of adult chickens following hypophysectomy by the transbuccal method has been found, in my own experience, to be comparatively low, though not as low as has been reported by Hill, Corkill and Parkes (1934). The first week after hypophysectomy, in particular, is the period of most severe losses. Since the trauma associated with the operation seemed to be primarily responsible for these losses a less traumatic technique was looked for; this was found in the oral approach² to the pituitary, which White (1933) used successfully on rabbits. This approach makes for a faster, simpler, and cleaner operation than the one using the transbuccal approach. Early mortality (in the first to second postoperative weeks) was considerably less (Rothchild, 1948) than that following the transbuccal method, although total mortality was practically the same by both methods.

The details of the method follow:

Equipment. The operating board is shown in perspective view in figure 1, and with its legend, is self-explanatory. The instruments needed (in addition to a dental drill, #8 or #10 excavating burrs, suction cannulas, and a suction pump) are a pair each of fine straight, and fine curved forceps, a small probe, a bone scraper (such as a dental discoid excavator), a pair of spring steel spreaders (shown in use in figure 2), a dissecting probe with end hooked and sharpened, and a spatula about 1 mm. in width.

Anesthesia. Nembutal (6.0% in 10% alcohol) by the intravenous route is used as the sole anesthetic. The effective dose is about 0.35 cc. per kilogram of body weight. Slightly over half of the total amount required should be given in a single quick thrust of the plunger and the remainder in 0.05 cc. amounts, with the speed of administration roughly in proportion to body weight. The best criterion of adequate anesthesia is a slight head twitch on pinching the comb. The last 0.10-0.15 cc. should in all cases be given slowly and carefully, with comb pinches between each 0.05 cc.

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² The oral approach was evidently also used by Mitchell (1929), but he gives no details about it.

The operation. The bird is laid, back down, on the operating board, the comb fitted into the comb slot, the neck between the upright prongs of the head holder, and the upper half of the beak into the beak holder (see figures 1 and 2). The bird's head is adjusted to a horizontal position, the rubber band is slipped under the lower jaw, with the tongue elevator under the tongue, and then fixed to the ap-

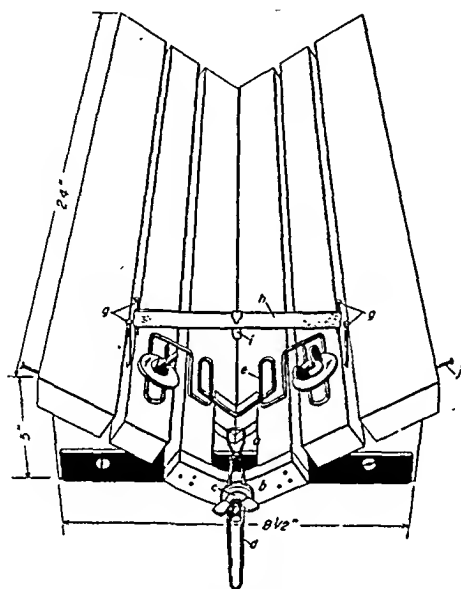


FIG. 1. Perspective view of the operating board for oral hypophysectomy: *a*—comb slot ($3 \times \frac{3}{4}$ inches); *b*—V-shaped piece of wood closing forward end of comb slot and serving as support for *c*—wing nut, bolt and washer; *d*—beak holder; *e*—head holder (made of heavy copper wire—the height of the upright prongs should be about $1\frac{1}{4}$ inches); *f*—wing nut, washer and bolt for clamping lateral arms of the head holder; *g*—pegs or nails for supporting *h*—rubber band for retraction of the lower jaw; *i*—wing or leg band fixed to forward half of the rubber band to serve as tongue elevator; *j*—nails to which cannula and suction tube can be attached during operation. The rear ledge (formed from the forward upright) on which a head block for young chicks rests can be seen through the comb slot. Most of the comb slot should be forward of this ledge. Overall dimensions of the board are as shown, and a section of the board in use is shown in figure 2. (Drawn from a photograph.)

propriate pair of pegs or nails. The anterior half, at least, of the cleft in the "soft palate" should be clearly visible. The cleft is spread with the pair of fine curved forceps. The ridge of tissue connecting the anterior edges of the cleft is cut gently with the bone scraper, and immediately afterward the bottom of the cleft is scraped clean with the bone scraper; the bone appears as a glistening white area containing a small, crescent-shaped ridge enclosing a tiny, cup-shaped depression (figure 2).

Blood, mucus, or regurgitated crop contents, if present, are removed with the suction cannula, and the spreaders are inserted (figure 2). The head of the bird should now be tilted as far forward as possible without permitting the lower jaw to obscure the operating field. Drill-

ing is started directly on the cup-shaped depression, and continued until the hard bone has been pierced. A second hole is then started anterior to the first by a distance approximately equal to the diameter of the burr. The two drill holes should fuse, the opening be extended laterally as much as possible, and the drilling continued at an angle of about 45° from the vertical. Repeated lifting and repressing the revolving burr against the bone will help the operator to recognize the characteristic sudden "give" that signals the completion of the hole. Reference to a sagittally cut preparation of the skull of the chicken will also be of considerable help in learning the landmarks, especially the depth of the bone.

The bottom of the hole is cleaned with probe and suction cannula, and enlarged if necessary. The appearance of the pituitary and its relationship to the circle of Willis are adequately described by Mitchell (1929) and need not be gone into here. After cutting the dura with the hooked dissecting needle, gentle application of the tip of the suction cannula to the surface of the pituitary capsule will usually bring the gland away without difficulty. The bleeding that follows is stopped temporarily with cotton wool; the drill hole is then refilled with bone wax, the spreaders and rubber band removed, and the bird allowed to recover completely from the anesthetic before being replaced in its cage.

Miscellaneous Notes. Operating time. Operating time can be as little as 3 minutes, and need not be more than 10. The major factors that lengthen operating time are bleeding at the bottom or sides of the cleft in the "soft palate" misplaced drilling, and hemorrhage at the end of drilling, or on cutting the dura with the hooked needle. All of these can be avoided or minimized with practice. *Use of atropine.* Hill (1934) mentions the use of atropine to control excessive mucus secretion in the mouth. However, I have found that, without atropine, occasional aspiration of the accumulated mucus with the suction cannula keeps the operating field adequately unobstructed.

Hypophysectomy of chicks. The bird illustrated in figure 2 is a chicken about 10-15 weeks old. The operating board shown in figure 1 has been modified for use with chicks by filling the comb slot with a wooden block, using a beak holder that can be lowered to a greater extent than the one used for adults, and a rubber band retractor for the lower jaw that is about one-half as wide as that used for adults. These simple modifications make the board as easy to use for chicks as for adults.

Removal of the posterior pituitary. The term *hypophysectomy*, as used here and elsewhere in work coming from this laboratory (Rothchild, 1946, a, b, c.; 1947; Rothchild and Fraps, 1947) refers to removal of the anterior pituitary only. Removals of the posterior pituitary have been attempted only in some unpublished experiments dealing with studies on the physiology of oviposition. The posterior pituitary was burned away with a cold cautery (the Birtcher "Hyfre-

cator") after the anterior pituitary had been removed. A fine bore suction cannula was inserted into the drill hole, together with the cautery electrode, and served to keep the hole free of blood during the cauterization.

Survival. A detailed report of the survival of adult hens following hypophysectomy by this and the previously used transbuccal technique is given in the paper accompanying this one (Rotnchild, 1948). In this place it will be sufficient to mention that mortality in the first week following oral hypophysectomy amounted to 12% (5 out of 42 birds), compared with an average value of 40% (52 out of 130 birds) following the transbuccal technique.

SUMMARY

A method of hypophysectomizing chickens is described, the main feature of which is the employment of the open mouth as the route by which the floor of the skull is reached. The advantages of this method over the one used previously (incision through the floor of the mouth) are its simplicity, cleanliness, and reduction in amount of time required and trauma induced.

ACKNOWLEDGMENTS

I wish to express my deepest thanks to Mr. Walter Stenhouse, the B.A.I. photographer, and to Mr. Henry Steuler of the Office of Information, U.S.D.A., for the drawings used in this paper.

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NOTES ON SURVIVAL AND BODY WEIGHT CHANGES OF ADULT HENS FOLLOWING HYPOPHYSECTOMY¹

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INTRODUCTION

PUBLISHED information on the survival of chickens after hypophysectomy is contained in only 3 papers. Two of these deal with immature chickens (Mitchell, 1929; Nalbandov and Card, 1943), and one with adults (Hill, Corkill and Parkes, 1934). In Mitchell's study, 160 Brown Leghorn chicks ranging in age from 3 weeks to 5 months (the majority between 3-6 weeks of age) were hypophysectomized, and all in which complete removal of the gland was effected died within 2 weeks. Nalbandov and Card (1943) made a study of 60 chicks operated on at 61 days of age. Seven of these died almost immediately postoperatively, and 16 were found to have been incompletely hypophysectomized. Of the remaining 37 birds, 70% (26) died within the first 30 days, and a total of 86% within the first 60 days after operation. Hill, et al. (1934) studied survival in 30 untreated completely hypophysectomized adult chickens, and found that 24 died within the first 48 hours after operation, and the remainder by the 31st day after operation.

Some of the short-time studies from this laboratory on adult hypophysectomized chickens had indicated for some time that early mortality, though high, was not at all comparable with that obtained by Hill, et al. Since relatively long-time studies with hypophysectomized adult hens were contemplated, it was decided to obtain a more complete picture of total survival time in the absence of replacement therapy, and if possible, of the factors (such as body weight changes) that are associated with, or influence it. These studies are reported in this paper.

METHOD AND MATERIALS

Operative procedures. Two methods of hypophysectomy were used. The transbuccal technique, which has been described in detail by Hill and Parkes

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¹ A preliminary report of this study was presented at the annual meeting of the American Society of Zoologists (Rothchild, 1944).

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(1934), was used on 130 birds; the modification of this method using an oral approach to the pituitary (Rothchild, 1948) was used on 42 birds. The differences in survival time between the two groups are presented and discussed in the following section. In referring to the two methods from this point on, the *transbuccal* method is used for Hill's technique, and the *oral* method for my modification of it.

Birds used. White Leghorn and Rhode Island Red hens only were used. One hundred and eleven of the birds operated on by the transbuccal method were 1 to 2 years old; the 42 birds operated on by the oral method and 19 of the birds operated on by the transbuccal method (serving as controls to the oral method) were 1.5 years of age. The birds were in good health at the time of operation, but not necessarily in laying condition.

Maintenance conditions. No therapy of any kind was given after operation, the birds being returned to their cages and kept under exactly the same conditions that existed prior to operation. All birds were housed in individual cages in laying batteries, and allowed *ad libitum* access to a standard laying mash and water. The windows were open to daylight. Electric lights controlled by an automatic switch were turned on every day of the year at 6:00 A.M. and off at 8:00 P.M., so that a minimum of 14 hours of light was maintained all year round. Temperature was not controlled, except by regulating the extent of window and radiator valve openings.

Observations. Time of death was reckoned by days or weeks, the day after hypophysectomy being considered as Day 1. The birds were allowed to die spontaneously, except in a few instances in which obvious signs of approaching death were present near the close of a working day. In these cases, the birds were killed and autopsied immediately, the day of autopsy being taken as the day of anticipated death. Body weight was observed several times during the first postoperative week, then at biweekly or weekly intervals for the next 9 weeks, and at weekly or longer intervals thereafter. Observations on molt and on the weights of several internal organs were also made but are not described in this paper.

Completeness of hypophysectomy. A safety bottle was attached to the suction line, and following each operation, the line itself and the bottle were washed with tap water, and the contents of the bottle examined carefully for the removed pituitary. Notes were made on each bird's protocol sheet as to how the gland was removed, i.e., in one intact piece, in two pieces, in fragments, or not seen. In addition, at autopsy each bird's sella was examined under a pair of dissecting binoculars. In 9 cases of long-time survival (see below), one of which died in the 27th postoperative week, and the other S killed between the 23rd and 70th postoperative weeks, serial sections of the sellae were made,³ and examined for pituitary rests.

In every one of the cases of spontaneous death, the pituitary was observed to have been removed in one piece at operation and/or the sella was seen to be bare of visible remnants at autopsy. Of the 9 long-time survivors whose sellae were serially sectioned, microscopic remnants of possible pituitary tissue, amounting to an insignificant fraction of the total gland, were observed in 5. Plumage, head furnishing, and body weight changes and

³ I am indebted to the Pathology Section of the National Cancer Institute, Bethesda Maryland, for the preparation of these sections.

extent of regression of the gonads, adrenals and thyroids of these birds differed in no appreciable respect from those seen in the long-time surviving birds that died spontaneously. I feel, therefore, that long time survival is not primarily related to pituitary rests, but is to be considered as part of the general pattern of survival following complete hypophysectomy.

RESULTS

Mortality

A. *Mortality following transbuccal hypophysectomy.* The numbers of deaths and mortality rates (percentage of survivors past a given period that died in the succeeding period) per week after hypophysectomy are shown in Table 1. As noted here, 21 of the 111 birds in this

TABLE 1. NUMBER OF BIRDS DYING OUT OF THE ORIGINAL 111, AND EQUIVALENT MORTALITY RATES* PER WEEK AFTER TRANSBUCCAL HYPOPHYSECTOMY

Number weeks after hypophysectomy	Number birds dying	Mortality rate*
1	46	41.5
2	10	15.5
3	4	7.5
4	6	12.0
5	9	20.0
6	7	19.5
7	5	17.0
8	1	4.0
9**	2	8.5

* Percentage of survivors past a given period that died in the succeeding period.

** 21 birds lived after the 9th week (see text).

series lived past the 9th week. Of these one each died in the 27th, 31st and 34th postoperative weeks, two in the 35th postoperative week, and one each in the 38th, 44th, 45th, 58th, 63rd, 82nd, 85th and 88th weeks. Eight birds in perfectly good health were killed in order to obtain their sellae for serial sectioning, and for other reasons.⁴ At the time of autopsy two were in the 23rd, 3 were in the 59th, and one each in the 60th, 64th and 70th postoperative weeks. (See preceding section on completeness of hypophysectomy.)

There was a possibly significant difference in the distribution of deaths within the first 9 weeks in relation to age, although both yearlings (60 birds) and 2-year olds (51 birds) showed approximately the same total mortality for this period (yearlings: 81.7%; 2-year olds: 80.4%). In the first 2 weeks after hypophysectomy 63% (38) of the yearlings, but only 35% (18) of the 2-year olds died; between the 3rd and the 9th weeks, however, 50% (11) of the surviving yearlings, and 70% (23) of the surviving 2-year olds died.

The first 3 days of the first week after hypophysectomy was in

⁴ Four of these birds were killed in order to obtain their thyroids and adrenals for cytological study by Dr. F. Payne, of Indiana University.

TABLE 2. NUMBER OF DEATHS AND THE EQUIVALENT MORTALITY RATES* PER DAY OF THE FIRST WEEK AFTER TRANSBUCCAL HYPOPHYSECTOMY (111 BIRDS STARTED)

Number of days after hypophysectomy	Number birds dying	Mortality rate*
1	14	12.6
2	12	12.3
3	10	11.8
4	2	2.6
5	3	4.1
6	4	5.7
7	1	1.5

* Percentage of the survivors past a given period that dies in the succeeding period.

general the period of most severe losses (Table 2). This period becomes of special interest in comparison with the equivalent period following hypophysectomy by the oral method (see below).

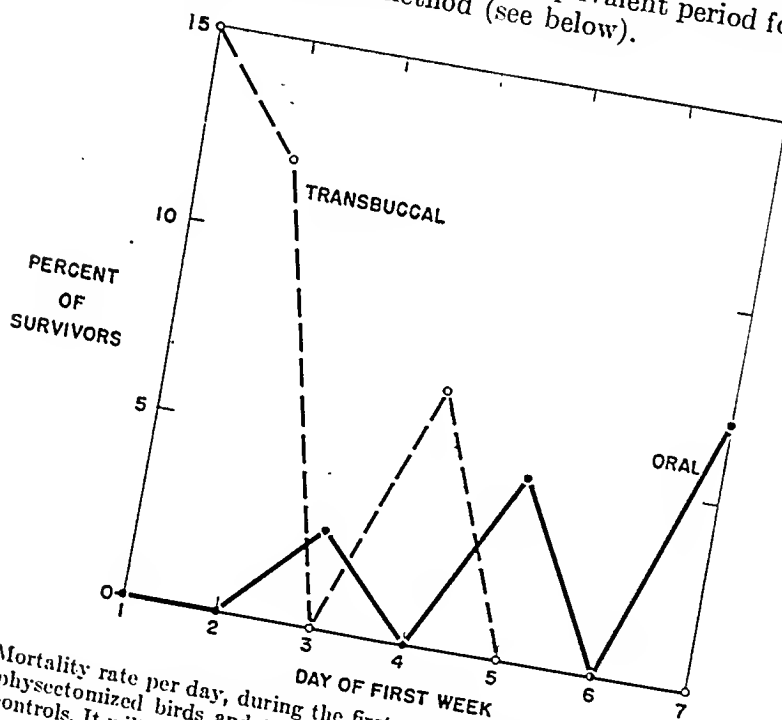


Fig. 1. Mortality rate per day, during the first week after hypophysectomy, of 42 orally hypophysectomized birds and 19 simultaneously run transbuccally hypophysectomized controls. It will be noted that the distribution of deaths following transbuccal hypophysectomy in this small series is of the same order as it is in the main series (111 birds) of transbuccal hypophysectomies (see Table 2).

B. Mortality following oral hypophysectomy. Of the 42 birds completely hypophysectomized by the oral method, the numbers dying per successive week from the first to 10th were as follows: 5, 3, 11, 7, 3, 6, 2, 1, 1, and 1. Mortality rate for the first postoperative week was thus only 11.9%.

In contrast to this, in a series of 19 control birds, hypophysectomized by the transbuccal method at the same time as those in the oral series, the numbers lost per successive week from the first to the 9th were as follows:

6, 0, 6, 3, 0, 0, 1, 2, and 1.

The mortality rate in the first postoperative week in this group of birds was thus more than 2.5 times that of the oral series. The distribution of deaths within the first week was also quite different in the two groups (figure 1). Total mortality, however, was practically the same, and was somewhat higher than in the series of 111 transbuccal hy-

TABLE 3. ACCUMULATED PERCENTAGE MORTALITY IN THE FIRST 10 WEEKS AFTER TRANSBUCCAL AND ORAL HYPOPHYSECTOMY

Number weeks after hypophysectomy	Transbuccal hypophysectomy (130 birds started)		Oral hypophysectomy (42 birds started)	
	Number birds dead at end of week	Percent of original number dead at end of week	Number birds dead at end of week	Percent of original number dead at end of week
1	52	40	5	12
2	62	48	8	14
3	72	55	19	45
4	81	62	26	62
5	90	68	29	70
6	97	74	35	83
7	103	78	37	90
8	106	81	38	90
9	109	83	39	93
10	109	83	40	95

pophysectomies described above. The differences in early mortality between all transbuccally and orally hypophysectomized birds and the similarities in total mortality are shown in Table 3.

Body Weight Changes

The body weight changes of each of the 172 birds used in this study were plotted individually, and examined carefully for evidence of a consistent pattern or patterns. The following general statements apply to all birds, regardless of the method of hypophysectomy.

1. During the first 3 to 4 days following hypophysectomy there was a practically universal drop in body weight; in about 78% of the birds this continued throughout all the first week.

2. There was a tendency for the body weight to become stabilized at about 10–15% less than the original body weight toward the end of the first week; the stabilization of weight continued, barring death, until the end of the 9th week (figures 2 and 3).

3. Death within the first 9 weeks was preceded by a fall in body weight from the stabilized level (see paragraph 2 above), if one had been reached, or by a continued fall from the original body weight.

4. Survival past the 9th or 10th week was accompanied by a rise

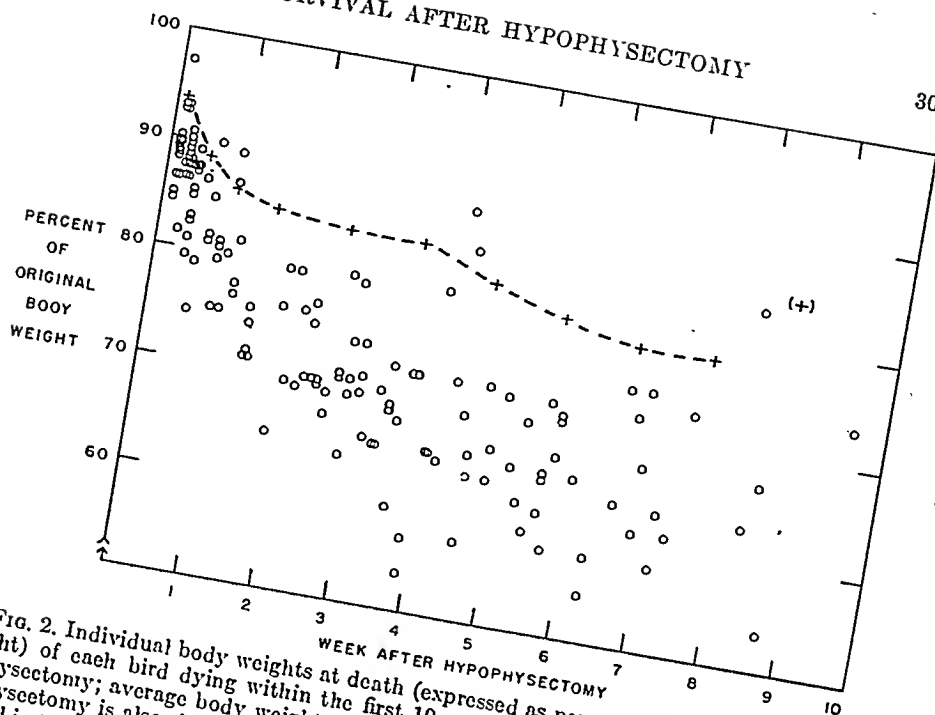


FIG. 2. Individual body weights at death (expressed as percentage of original body weight) of each bird dying within the first 10 weeks after transbuccal and oral hypophysectomy; average body weight of these short-time survivors per week after hypophysectomy is also shown (broken line and +). Body weights at death are not included in the average body weights. Average body weight shown at week 9 is placed in parentheses since only 1 bird was present in this group.

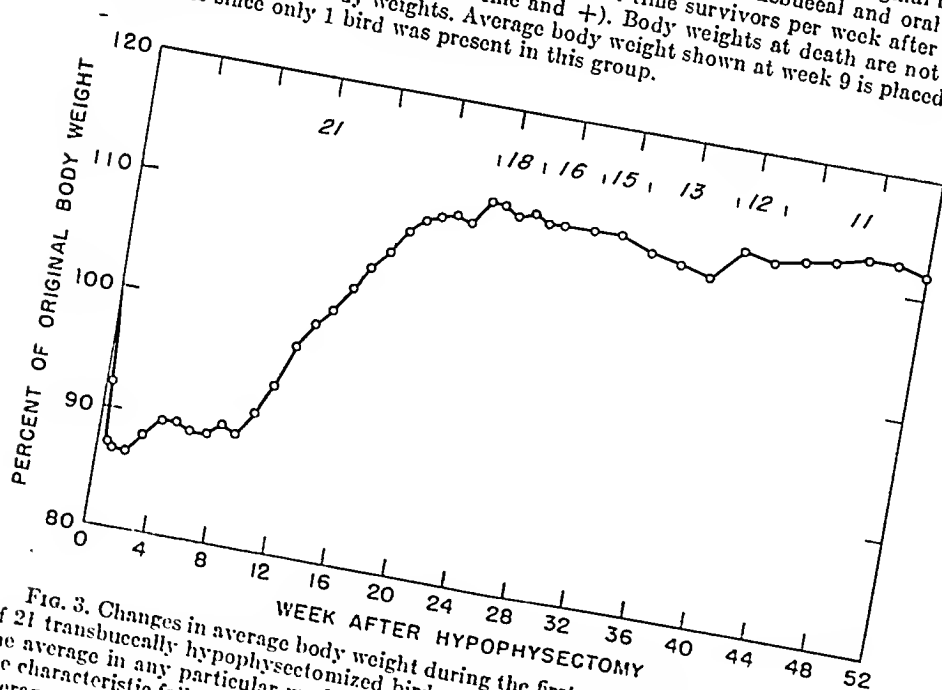


FIG. 3. Changes in average body weight during the first year after hypophysectomy of 21 transbuccally hypophysectomized birds surviving past the 9th week. Values for the average in any particular week do not include the body weights of birds showing the characteristic fall preceding death (see text). The numbers of birds included in the average are shown above the curve.

in body weight, starting roughly with the 10th week, and continuing until the 23rd week (figure 3). From this point on body weight again remained stable at about 111% of the original body weight for as long as the bird lived, but death within this period was also preceded by a fall in body weight.

5. The total amount of body weight lost up to the time of death (expressed as per cent of original body weight) increased throughout the first 30 days after hypophysectomy, and then remained fairly constant, although the amount of variation around the apparent average was quite large (figure 2).

DISCUSSION

The primary intent of the studies described in the preceding pages was to establish the general picture of survival time of adult hens, maintained without therapy of any kind, after hypophysectomy. From this standpoint, the data presented are probably as satisfactory as any. They establish the fact that a definite pattern of survival takes place. This pattern has three fairly clearly defined characteristics; a period of relatively high mortality embracing the first post-operative week and followed by a period of reduced mortality; a second period of relatively severe losses extending from about the 3rd to the 7th week, in which a major portion of all losses occur; and a period of long time survival of birds that live past the 9th-10th post-operative week. The data presented should also dispel the belief that has existed since R. T. Hill's work was done that the adult hen is unsuitable for studies involving hypophysectomy because of the excessive losses within the first 48 hours. The oral technique for hypophysectomy permits well over the majority of the birds to live without replacement therapy for about 2 weeks after operation, and there is ample time within this period for the execution of a variety of studies on the physiology of the avian pituitary.

Although the primary purpose of this study has been accomplished many interesting questions have been raised by it. The reduced mortality in the early period following oral hypophysectomy as compared with that obtained with transbuccal hypophysectomy, and the importance of this difference for determining the cause of early postoperative death; the difference in the distribution of deaths in relation to age; the cause of death in the 4th to the 7th week, and the relationship between deaths in this period and the failure to maintain a stable body weight; the fact that survival past the 9th week is practically synonymous with long-time survival, and the correlation between long-time survival and increase in body weight are some of the most interesting of these.

Some light is thrown on the question of better early survival following oral hypophysectomy by data gathered by Dr. A. Nalbandov of the University of Illinois (personal communication). He found that hypophysectomized chicks subjected to repeated short periods

of illumination, in addition to regular daylight, fed and survived much better than their controls, maintained under regular daylight alone. My orally hypophysectomized birds showed a slightly, but consistently smaller loss of body weight in the first 3 days following operation than did the transbuccally hypophysectomized birds, so it is definitely possible that better feeding contributed to the reduction of early mortality in these birds, in the same way that it contributed to the better survival of Nalbandov's chicks. The factor of reduced trauma is unquestionably present, but unfortunately, none of the data accumulated so far are conclusive enough for a clear-cut differentiation between better feeding and reduced trauma as the primary factors responsible for reduced early mortality. The same holds true for the other questions raised by this study, and it would therefore be pointless to discuss them at this time.

SUMMARY

Following hypophysectomy, adult hens tended to die most frequently in two main periods: the first week after hypophysectomy, and between the 4th-7th weeks. Roughly 80% of all birds started died by the end of the 9th week, but those remaining lived for comparatively long periods of time (27 to 85 weeks after hypophysectomy). Losses in body weight took place in practically all birds during part or all of the first postoperative week, but in the succeeding weeks up to about the 10th, a tendency for stabilization of body weight at roughly 85% of the original body weight was shown by most birds. Birds surviving past the 10th week showed a steady increase in body weight between the 10th and 25th weeks, and a stabilization of body weight at an average value of 111% of the original weight for as long as the bird lived. Death, however, at any time after hypophysectomy was preceded by a sharp drop in body weight. Hypophysectomy was performed by two methods: using the transbuccal approach to the floor of the skull (incision through the floor of the mouth), and using the oral approach (through the open mouth). The oral approach was followed by a considerably reduced mortality in the early postoperative period, but total mortality was practically the same following either method.

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THYROGLOBULIN FORMATION IN THE THYROID FOLLICLE VISUALIZED BY THE "COATED AUTOGRAPH" TECHNIQUE

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THE UNIQUE histological structure of the thyroid has prompted a number of attempts to relate its cytological features to the process of hormone formation. However, the theories on the subject (cf. Bargmann, 1939) have left many, so far unanswered questions. For example, do the cells secrete into the circulation or in the direction of the colloid? Does this colloid merely represent the site of storage of excess thyroid hormone or does it in fact play a role in the formation of the thyroid principle? These and similar histophysiological points have remained in doubt, presumably because of the limitations of classical histology.

The use of radioactive isotopes of iodine offered a new approach to these problems. Radio-iodine may be located in sections of thyroid gland by the autographic method, which takes advantage of the action of radio-elements on a photographic emulsion. A review of the autographic results obtained to date (Gross and Leblond, 1947) showed that, within 24 hours after administration, radio-iodine was organically bound in the colloid of the thyroid follicle, both in resting and stimulated glands. However, the resolution of the earlier autographs was poor. It was, therefore, decided to reexamine the entry of radio-iodine into the thyroid of the rat by the improved "coated autograph" technique (Bélanger and Leblond, 1946; Leblond, Percival and Gross, 1948), under carefully controlled conditions of iodine intake, using intact and hypophysectomized rats. The results as reported below clarified several problems of thyroid histophysiology.

METHODS

Two similar series of experiments were carried out with essentially the same results; but only the latter will be reported in detail.

Three month old male albino rats were separated into three groups, in one of which the animals were hypophysectomized. One month later, all the animals were placed on Remington's low iodine diet (Levine, Remington and von Kolnitz, 1933) to which 5% yeast had been added. One of the two intact groups received a subcutaneous injection of 20 micrograms of iodine per rat per day. Since the diet supplied about 2 micrograms per animal per day,

each animal in this group received a daily total of about 22 micrograms of iodine.

After 6 weeks on the diet, all the animals were given a single intraperitoneal dose of carrier-free "tracer" radio-iodine (I^{131}) as sodium iodide. In the group on the high iodine intake, the injection of the daily dose of I^{127} was discontinued 36 hours prior to the administration of radio-iodine. Each of the three groups was then divided into two subgroups, one of which was sacrificed one hour, and the other, 24 hours after the radio-iodine treatment. The left lobe of the thyroid gland was digested in 2N sodium hydroxide by heating in a water bath and then evaporated on a watch glass for measurement of the radioactivity with the Geiger counter. The count was doubled to obtain the approximate radio-iodine uptake by the 2 lobes of the thyroid gland (Table 1).

The right lobe was left attached to the trachea, fixed in Bouin's fluid and taken through dioxane for paraffin embedding. Histological sections were cut at 5μ , mounted and dried thoroughly on a 40° electric hot plate for 2 to 3 hours. Some sections of each gland were stained with hematoxylin-eosin, and some, with Masson's light green trichrome. The sections were then coated with celloidin and photographic emulsion as indicated elsewhere (Leblond, Percival and Gross, 1948). Exposure varied from one month in the case of the hypophysectomized animals, to two hours in the case of the intact animals receiving no iodine supplement.

In order to improve the sharpness of the localization of the radio-iodine, experiments were also carried out in which the photographic emulsion was diluted 8 times with a 1% solution of Duponol C (lauryl sulphate). Under these conditions, the thickness of the emulsion coating was much reduced and the diffusion of the photographic reaction decreased (Fig. 5).

Several accessory experiments were also performed. In order to compare the thyroid stimulating action of the I-deficient diet to that of the thyrotrophic hormone, guinea pigs weighing from 150 to 200 gm. were divided into an untreated control group and an experimental group given 4 units of thyrotrophic hormone (antuitrin-T) over a period of 4 days. Then all animals received an intraperitoneal injection of tracer radio-iodine. Their thyroids were autographed and counted as above (Table 3).

At the last step of this investigation, it became important to decide whether the organic iodine released by the thyroid cell was immediately released into the blood or into the colloid or simultaneously in both. For this purpose, the rate of accumulation of organic iodine in the thyroid and in the blood was compared. Eight rats weighing 150 ± 16 gm. were kept on the iodine-deficient diet for 6 weeks, and then divided into groups of two, of which one animal was left intact and the other one thyroidectomized just prior to injection. Following an intravenous injection of carrier-free radio-iodine, the groups were sacrificed 2, 7, 15 minutes and 24 hours later respectively. One thyroid lobe was autographed, the other was emulsified in saline and precipitated with an equal volume of Bouin's fluid. Both precipitate and supernatant were then counted. The blood of all animals was oxalated and precipitated with double volume of 10% trichloroacetic acid containing 20 micrograms of iodide per cc. The precipitate was washed twice with 5% trichloroacetic acid containing 10 micrograms of iodide per cc., dissolved in 0.5 N NaOH, re-

precipitated and washed as above. The supernatants and the washed precipitates were then counted under the Geiger tube (Table 4).

RESULTS

In the animals receiving a moderately high iodine intake, i.e., 20 micrograms subcutaneously daily, the thyroid follicles had a rather low epithelium. The colloid was variable in consistency and stained either red and/or green with the Masson trichrome. A fair radio-iodine

TABLE 1. THE FIXATION OF TRACER AMOUNTS OF RADIOACTIVE IODINE (I^{131}) IN THE THYROID UNDER VARIOUS EXPERIMENTAL CONDITIONS

Treatment	Approximate iodine intake (μ gm./day)	% injected dose 1 hr. after injection	24 hrs. after injection
Low iodine diet +20 μ gm. I daily	22	2.64	7.95
		2.71	16.82
		3.20	
		3.90	
		Average 3.11	Average 12.39
Low iodine diet +Hypophysectomy	2	0.23	0.41
		0.22	
		0.10	
		0.60	
		Average 0.29	Average 0.41
Low iodine diet Controls	2	33.10	72.00
		39.10	46.50
		52.20	58.10
		39.60	56.00
		Average 41.00	Average 58.15

uptake by these glands was shown with the help of the Geiger counter (Table 1). The distribution of the radio-iodine as localized on the autographs showed a definite pattern. One hour after the injection, the reactions were predominantly in the form of rings indicating the presence of iodine in the epithelium of the thyroid follicles (Figs. 1 and 3). In thin emulsion preparations viewed under high power, the individual silver granules indicative of the presence of radioactivity could be seen to occur in the cytoplasm but not in the nucleus. In the more columnar cells, the granules predominated in the apical region (Fig. 5); while in the lower cuboidal cells, the radioactivity extended throughout the cytoplasm. However, some of the follicles with a cellular reaction also had granules in the peripheral parts of the colloid. Finally, some of the more central follicles showed dot-like reactions indicating a diffusion of the radioactivity throughout the colloid (Fig. 3).

In order to estimate the size and relative numbers of the ring-like and dot-like reactions, projection drawings of unstained autographs were made at a magnification of 200 diameters. Strips through various parts of the gland were drawn at random, and a minimum of 350

follicles were counted in each section. The mean outer diameter of rings and dots was estimated by arbitrarily selecting their center, drawing the longest possible diameter and its perpendicular through each center, and averaging the length in millimeters of these two diameters. The mean inner diameter of the rings was similarly estimated by measuring two perpendicular diameters of the unreactive part (Table 2).

TABLE 2. PERCENTAGE OF FOLLICLES IN WHICH THE RADIOACTIVITY IS IN THE CELLS AT 1 HOUR AFTER INJECTION OF I^{131} (RATS GIVEN 22 MICROGRAMS OF IODINE DAILY)

Body of gland	% of reactions appearing as rings	Mean outer diameter of rings	Mean outer diameter of dots	Mean inner diameter of rings	Calculated % of follicles with unreactive center*
A	53				
B	55	12.4			
C	44	17.6	6.0	6.8	96
D	29	15.5	6.3	11.8	82
Isthmus		13.2	6.5	9.3	74
A	55	9.9	6.9	8.0	48
B	48				
C	54	11.7	5.7	5.3	102
D	63	16.4	5.9	6.8	83
Peripheral follicles		15.3	8.9	8.1	109
A	71		7.2	7.6	127
B	76	17.2			
C	67	18.1	6.5	11.0	110
D	76	17.1	6.5	12.7	108
		15.4	8.0	9.5	120
			7.4	9.1	128

* The calculated percentage of follicles with an unreactive center is equal to the percentage of reactions appearing as rings (1st column) multiplied by the ratio of the mean outer diameter of rings (2nd column) over the mean inner diameter of rings (4th column).

This formula is based on the assumption that the follicles are spheres. It can be shown that the ratio of the apparent to the true number of follicles with an unreactive center is equal to the ratio of the mean inner to the mean outer diameter of these follicles. The last value was not available and was replaced in the calculations by the mean outer diameter of the rings. Hence the results should be somewhat too high.

Since the production of a ring-like autograph resulted from the fact that the central portions of a follicle did not contain radioactivity, such a follicle was recognizable only when the plane of section went through the unreactive central portion. If the plane of section went through the cells of a follicle with an unreactive center, this follicle appeared as a dot. The estimated number of follicles producing a ring-like autograph will, therefore, be smaller than the true number of follicles with an unreactive center, but an approximation to the true figure may be calculated¹ (Table 2, column 5).

While the calculated results were too high, probably by 10 to 20%, the indication was that in almost all the follicles of the periphery and isthmus and in over half those of the center of the glands the radioactivity was present in the cells, while the remaining follicles of the

¹ Our thanks are due to Dr. J. Stanley, Department of Zoology, McGill University, for a mathematical study of this problem. No formal solution could be obtained.

inner regions of the gland had radioactivity extending throughout the colloid.

Twenty-four hours after injection of radio-iodine to the animals on the higher iodine intake, the autographic reactions extended to the whole colloid of practically all follicles (Figs. 2, 4 and 6). Indeed, most of the cells in these animals no longer contained radioactive material. It may be noted that the blackening of the colloid in the various follicles was quite variable, some of them showing an intense, and others, only a slight reaction (Figs. 4 and 13).

TABLE 3. THE FIXATION OF TRACER AMOUNTS OF RADIOACTIVE IODINE (I^{131}) IN THE GUINEA PIG THYROID AFTER THYROTROPHIN INJECTION

	Weight of the right thyroid lobe after fixation (mg.)	% of the injected dose found in the thyroids at 1 hr. after injection
Control	23	0.39
	31	0.83
	26	0.38
	34	0.53
	28	0.64
	15	0.37
	Average 26	Average 0.52
Thyrotrophin treated	23	3.87
	42	4.31
	46	5.19
	53	3.38
	Average 41	Average 4.19

In the hypophysectomized animals, the thyroid epithelium was flat, the lumen of the follicle, fairly large, and the colloid, rather dense and acidophilic. The radio-iodine uptake, estimated on the Geiger counter, was low (Table 1). At one hour after injection, the autographs showed the radioactive material mostly present in the cells (Fig. 7). At 24 hours, the radio-iodine was still present in the cells of most follicles, although a few follicles showed its penetration into the colloid (Fig. 8).

The thyroid glands of the control animals on a low iodine intake showed evidence of stimulation. The epithelium was increased in height; the follicular lumen was small and contained a rather thin colloid, which stained a pale green with the Masson trichrome. The radio-iodine uptake was considerable even at 1 hour after injection (Table 1). The autographs showed the radio-iodine consistently in the colloid at both one and 24 hours after injection (Figs. 9 and 12). Central collections of silver granules were found even in those follicles in which the lumen was very narrow and the colloid, so scanty as not to take up any stain. The absence of autographic reaction over the epithelium indicated that the amount of organically bound iodine present in the cells was very small or absent (Figs. 11 and 12).

Similar results were obtained with the thyroids of guinea pigs treated with thyrotrophic hormone. The radio-iodine uptake was increased (Table 3). Autographs showed the presence of the radioactivity in the colloid, not only the stimulated glands, but also in the normals.

In the experiments carried out soon after intravenous injection in iodine deficient rats, the autographs revealed the presence of radio-

TABLE 4. RADIO-IODINE (I^{131}) CONTENT OF THYROID GLAND AND TRICHLORACETIC PRECIPITATE OF BLOOD PLASMA (BLOOD PLASMA TAKEN AS 2.15% OF BODY WEIGHT)

Time after injection	Treatment	Thyroid % of inj. dose	Bovin precipitate of thyroid - % of total thyroid I^{131}	Trichloroacetic precipitate of blood plasma (% inj. dose)	Trichloroacetic precipitate of blood plasma (% total plasma I^{131})
2 minutes	thyroidectomized	—	—	—	—
7 minutes	intact	1.16	92	0.04	0.24
15 minutes	thyroidectomized	4.09	95	0.03	0.22
24 hours	intact	—	98	0.01	0.09
	thyroidectomized	11.65	99	0.01	0.14
	intact	52.50	—	0.02	0.09
				0.72	0.19
					78.50

* The values for the blood of the thyroidectomized animals were so low as to be within the error range of the Geiger counter background.

TABLE 5. RADIO-IODINE (I^{131}) CONTENT OF HALF-SATURATED AMMONIUM SULPHATE AND BOVIN'S FLUID PRECIPITATE OF THE THYROID GLAND

Time after injection	% of thyroidal I^{131} in Bovin precipitate	% of thyroidal I^{131} in ammon. sulph. precipitate
2 mins	96	96
7 mins	95	94
15 mins	96	93

activity in the colloid of all follicles at all time intervals. This was true even at 2 minutes after injection (Fig. 14). Quantitative measurement of the radioactivity (Table 4) showed that the thyroid glands fixed large amounts of radio-iodine. Over 90% of this iodine was precipitable with Bovin's fluid; the histological fixative used in making the autographs. However, only small quantities were observed in the trichloroacetic precipitates of plasma at 2, 7 and 15 minutes after injection. These were no greater in the normal than in the thyroidectomized animals and were therefore attributed to experimental error. In contrast, 78% of the blood radio-iodine was present in the plasma precipitate of the intact animal sacrificed twenty-four hours after radio-iodine treatment. Therefore, the deposition of organic radio-iodine in the thyroid gland preceded the appearance of organic radio-iodine in the blood.

In an attempt to identify this organic compound in the gland, thyroids from animals given radio-iodine as in the previous experi-

ment were homogenized in saline. This saline extract was divided into 2 aliquots, which were then precipitated with equal volumes of saturated ammonium sulphate and Bouin's fluid respectively (Table 5). Both reagents were equally effective, precipitating over 90% of the total thyroïdal iodine. Since half saturated ammonium sulphate is used to isolate thyroglobulin from the thyroid (Oswald, 1899; Rivière, Gautron and Thély, 1947), it was thus shown that the iodo-protein precipitated by Bouin's fluid was also thyroglobulin.

DISCUSSION

It should be emphasized that the amount of radioactive iodine administered to the animals consisted of a very small or "tracer" dose of iodide. This dose had an iodine content of about $0.01\mu\text{g.}$ or less, i.e., an amount of iodine too small to interfere with the normal metabolism of this element. The injected radio-iodide, mixing with the body iodide as it enters the circulation, will serve as an indicator of the behavior of iodide under physiological conditions.

Apparently the circulating iodide originates from two sources; the diet, and the breakdown of the iodinated products of the thyroid. Since a breakdown to iodide was observed after administration of diiodotyrosine (Leblond and Sue, 1942), thyroxine (Gross and Leblond, 1947, b) and even iodo-proteins (unpublished results), this is likely to take place with the thyroid hormone after it has been secreted. The supply of iodide from the diet seems to be fairly continuous since the rat stomach contains some food at any time of the day. Both sources of iodide should provide a relatively steady supply of iodide to the circulation. Therefore, while the radio-iodine reveals a sequence of events beginning at the time of injection, the relative constancy of the iodide supply is such that the sequence of events visualized with radio-iodine repeats itself indefinitely.

The radio-iodine producing the autographic reactions must be in a chemical form that is insoluble in the reagents used for routine histological processing. Since it must be neither water nor fat soluble, it seems unlikely that it exists as iodide, or in small organic molecules such as diiodotyrosine or thyroxine. This was demonstrated at least in the case of iodide and thyroxine, which may be extracted from the tissues by several of the reagents used in histological technique (unpublished experiments). On the other hand, proteins are usually precipitated during histological fixation and thus may be retained in histological sections. Thus it was found that the thyroïdal radio-iodine was precipitated equally well by both Bouin's fluid and half-saturated ammonium sulphate (Table 5). Since the latter reagent has been shown to precipitate almost all of the iodine of the thyroid as thyroglobulin (Oswald, 1899; Rivière, Gautron and Thély, 1947) it was assumed that the autographic reactions were due to the iodine present in this iodoprotein. Further, since short time intervals after injection were used, the autographs were taken to indicate the presence of *newly formed thyroglobulin*.

The sequence of events was most apparent in the moderately active glands of animals supplied with 22 micrograms of iodine daily. One hour after administration of radio-iodine, the newly formed thyroglobulin was present mostly in the epithelium of the thyroid follicle (Fig. 1). Therefore, the iodination of the proteins must have taken place in the cells, a fact in agreement with the finding in the epithelium of a peroxidase and oxidase which presumably may transform iodide into a form capable of iodinating proteins (Dempsey, 1944). Although the radioactive material seemed to be dispersed throughout the cytoplasm of the very flat cells, a clearcut localization in the apical region was visible in most other cells (Fig. 5), indicating that thyroglobulin is formed mainly in the cell apex. In our experience the same is true of colloid droplets which, when present, are located in the apex of the taller cells but are less definitely localized in lower cells. Since colloid droplets were quite rare in these sections, while the apical location of the newly formed thyroglobulin was of common occurrence the results may possibly be explained by the hypothesis that the cells contain antecedents of the colloid at or beyond the limit of microscopic visibility (Uhlenhuth, 1923; Gersh and Caspersson, 1940).

The demonstration of the sequence of events was completed by the results obtained in the 24-hour group, since at this time interval the new thyroglobulin was present in the colloid of all follicles. Obviously, the follicles which soon after injection contained the material in their cells had by now secreted it into the lumen.

In summary then, the iodide taken up from the blood by the thyroid cell reacted with the cellular proteins to yield thyroglobulin; this reaction predominating in the apical region of the cell. The iodinated protein was then rather quickly released into the colloid.

In hypophysectomized animals, the rate of formation of thyroglobulin was considerably decreased, since the radioactivity was still in the epithelium of most follicles at 24 hours after injection. It was pointed out earlier that the thyroid of the hypophysectomized rat retained the ability to collect iodide from the blood (Leblond and Sue, 1942). The thyroid cell was now found capable of building thyroglobulin and secreting it into the colloid in the absence of the hypophysis; but these phenomena took place at a much slower rate than normally.

On the other hand, the animals with stimulated thyroids, i.e., those on a low iodine intake, showed new thyroglobulin in their colloid as early as 1 hour and even 2 minutes after injection of radio-iodine (Fig. 14). Both the stimulated and resting thyroids of the guinea pig (Table 3) also showed a reaction in the colloid at 1 hour after injection. As pointed out above, these results did not necessarily mean that radioactive thyroglobulin was absent in the cells, but only that it was definitely less concentrated there than in the colloid. It is thus possible that the iodination of the proteins, and especially the deposition of the iodinated thyroglobulin into the col-

loid occurred so rapidly that at no time after injection the cells contained sufficient radioactive material to give a clearcut autographic reaction.² However, the results did not eliminate the less likely possibility that cells in the stimulated thyroids merely collected iodide from the blood to deposit it into the colloid where iodination would take place. At any rate, in stimulated thyroids as in the more resting glands of the iodide treated and hypophysectomized animals, the end result of the process was the accumulation of radioactive thyroglobulin in the colloid.

The evidence presented above indicated a definite polarity of the thyroid cell in its metabolism of iodine: that is to say, iodide entered the cell from the circulation, and was deposited as iodinated protein into the lumen of the follicle. This orientation of the thyroid cell toward the colloid was in agreement with the usual morphological criteria that Golgi apparatus, secretions droplets, cytocentrum and terminal bars are located between the nucleus and the secretory pole of the cell. Thus, it has been shown that terminal bars and cytocentrum were close to the membrane separating the cell from the colloid (Cowdry, 1921; Wahlberg, 1933; Bargmann, 1939). Furthermore, with the exception of flat cells, where a clearcut localization was not apparent, it was our experience as well as those of most authors to find the Golgi apparatus between the nucleus and the lumen (Okkels, 1931; Bargmann, 1939). The same might be said to colloid droplets which, in addition, increased in size as they came closer to the inner cell membrane.³

A careful examination of the slides obtained from the iodine-treated and control animals revealed that *all* follicles had formed some thyroglobulin as early as 1 hour after administration of radio-iodine. Similar observations were made under various experimental conditions in rats as well as in guinea pig, mouse, rabbit, man, etc. . . . (unpublished results). The only exceptions encountered were in old individuals and in hypophysectomized animals in whom some of the follicles had not fixed a detectable amount of radio-iodine after this time interval. In both cases, the extreme slowing down of the gland activity was presumably the cause of the lack of uptake. At any rate, the entry of tracer iodine in all follicles of young adult rats within 1 hour after administration indicated that *at all times all follicles were active in building up thyroglobulin and depositing it into the colloid*.

This very simple observation made it possible to eliminate the theories of thyroid function based on a "reversal" of the activity of

² The laws of biological precursors (Zilversmit, Entenman and Fishler, 1943) require that the bound iodine in the cells, being precursor of that in the colloid, have a higher specific activity. This condition may be fulfilled even with a low concentration of radioactivity in the cells provided their I^{127} content is low enough in relation to that of the colloid.

The presence of iodine in the epithelium has been shown by Gersh and Caspersson (1940).

³ Biondi (1892); Uhlenhuth (1923); Severinghaus (1933); Wahlberg (1933); de Robertis (1941a, 1942); Grasso (1946); Dvoskin (1947); etc. . . .

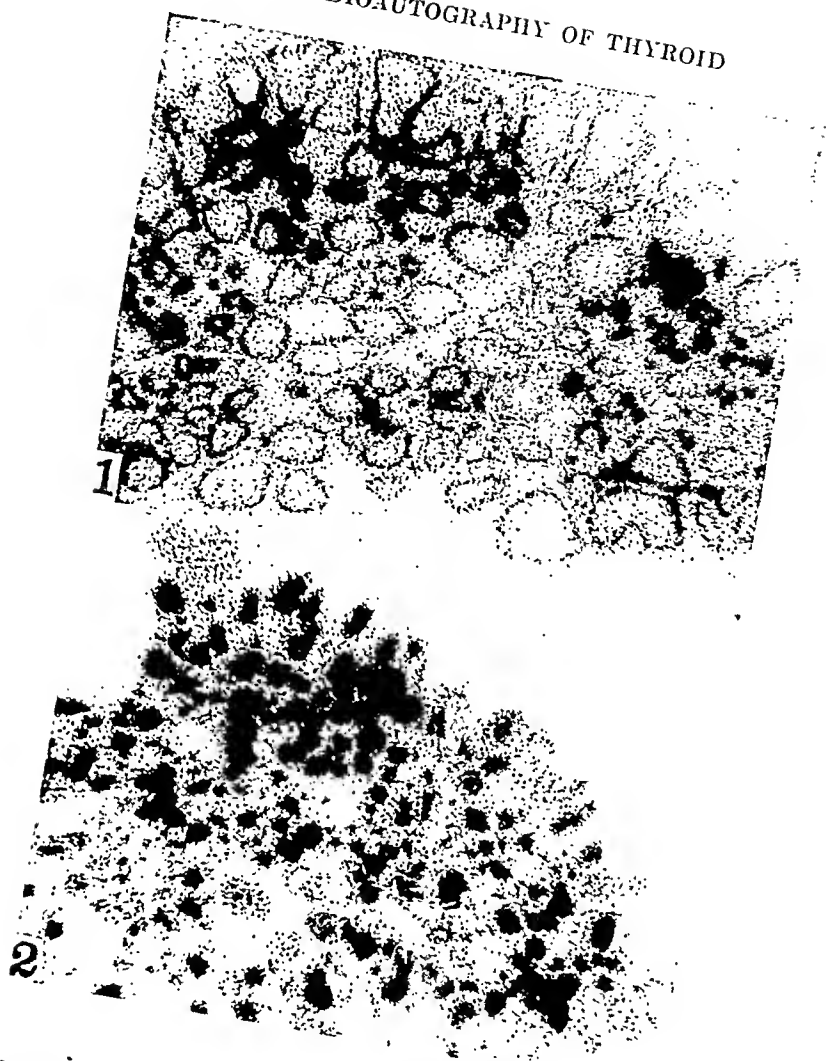


PLATE I

FIG. 1. Unstained coated autograph of thyroid from rat treated with 20 micrograms of iodine daily. The animal was sacrificed one hour after injection of radio-iodine (Animal B, table 2). Most follicles appear as rings because of the deposition of photographic granules at the level of the epithelium. $\times 65$

FIG. 2. Unstained coated autograph of thyroid from rat treated with 20 micrograms of iodine daily. The animal was sacrificed 24 hours after injection of radio-iodine. Practically all follicles appear as dots, because of the deposition of photographic granules over the colloid. $\times 65$.

the follicle. According to the reversal hypothesis, the cells of a follicle are capable of secreting in the direction of the colloid at one time and, at other times, reabsorbing the colloid to secrete it into the extracellular spaces and blood vessels.⁴ This theory derived support from Cow-

⁴Severinghaus (1933); Gillman (1934); Thomas (1934); Fasella (1936); Williams (1937, 1939); Fevel and Varangot (1938); de Robertis (1942). Other references may be found in Bargmann (1939).

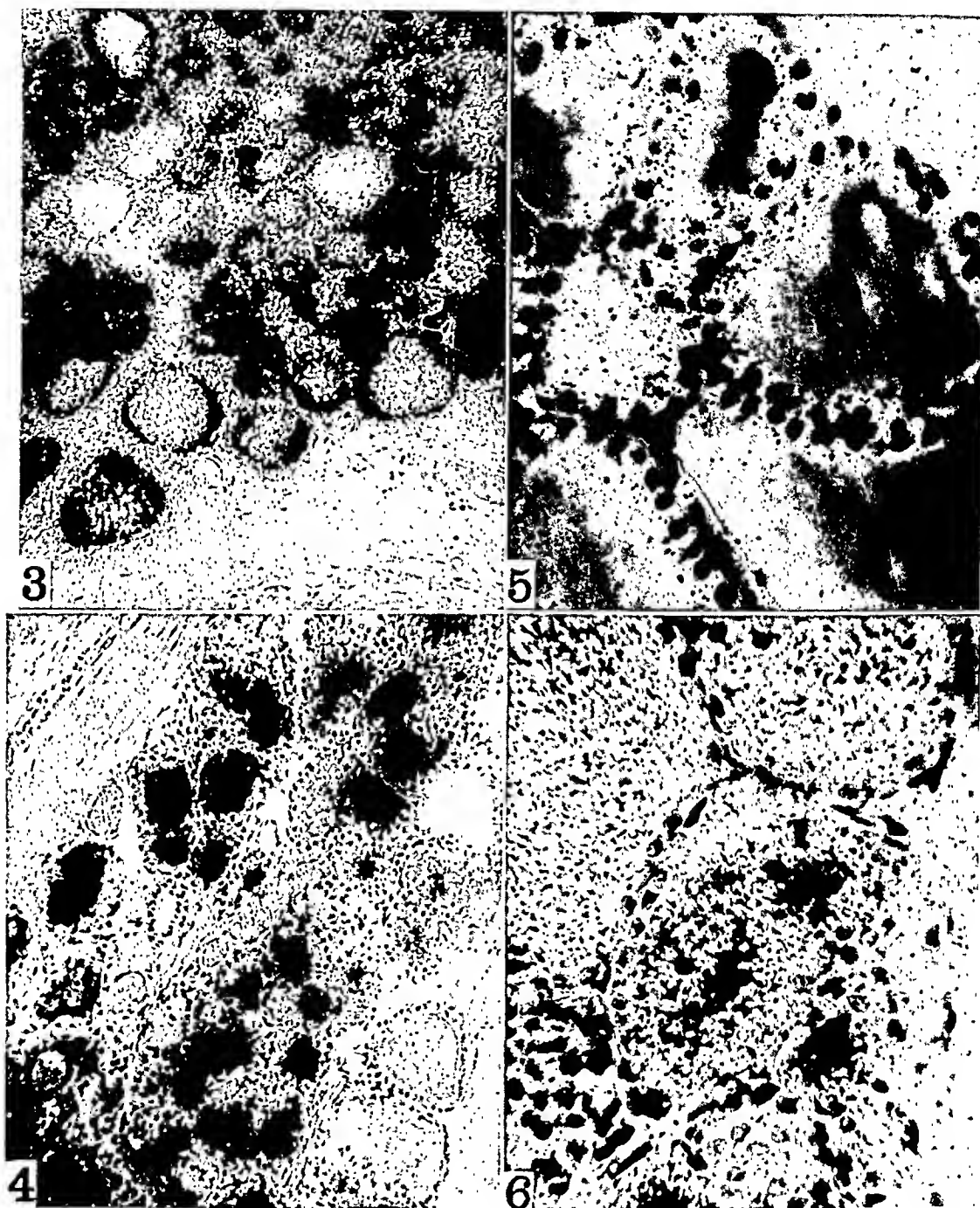


PLATE 2

H. E. stained coated autographs of thyroids from rat treated with 20 micrograms of iodine daily.

FIG. 3. The animal was sacrificed 1 hour after injection of radio-iodine (Animal C, table 2). Note both rings and dots. $\times 125$.

FIG. 4. The animal was sacrificed 24 hours after injection of radio-iodine. The photographic reaction is present over the colloid, although its intensity varies greatly in the different follicles. $\times 125$.

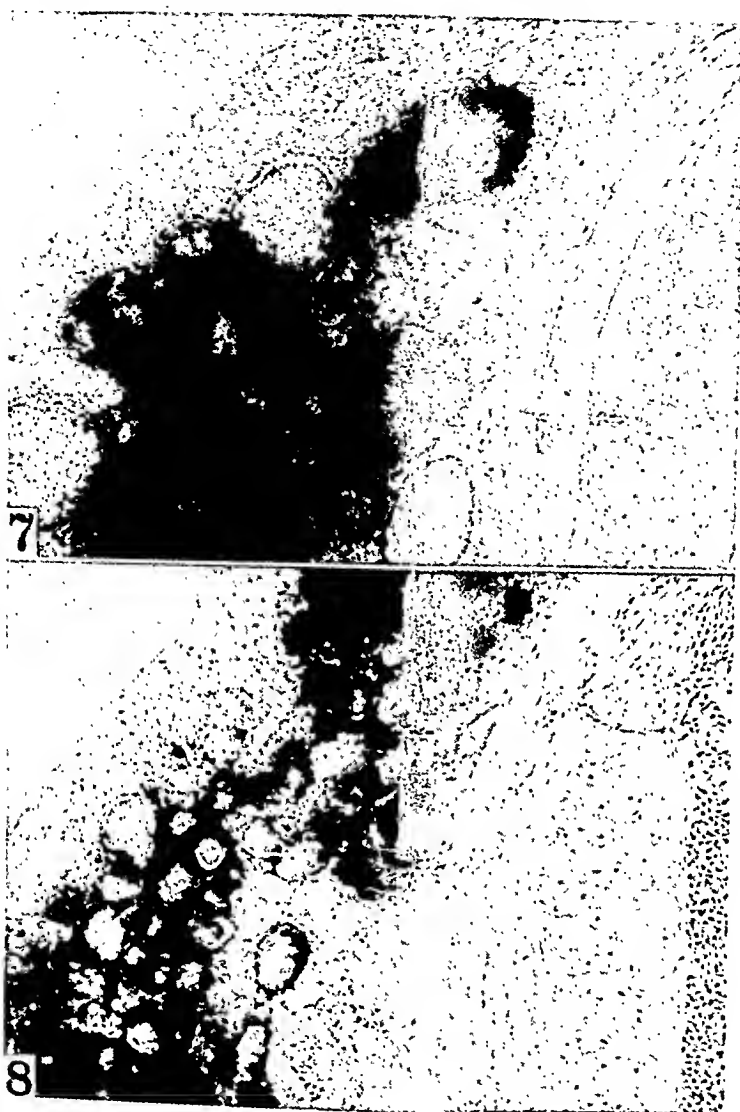


PLATE 3

11. E. stained coated autographs of thyroid from hypophysectomized rats on iodine deficient diet.

Fig. 7. The animal was sacrificed 1 hour after radio-iodine injection. The cells are thin and show a variable reaction. No reaction is apparent in the colloid. $\times 125$.

Fig. 8. The animal was sacrificed 24 hours after radio-iodine injection. The reaction is still cellular in most follicles, although a few follicles in the upper part of the picture show the radio-iodine throughout the colloid. $\times 125$.

Fig. 5. The animal was sacrificed 1 hour after injection of radio-iodine. A thin emulsion was used (Animal B, table 2). The amount of background fog may be estimated from the black granules in the right upper corner. A significant reaction occurs in the apical region of the cells. $\times 400$.

Fig. 6. The animal was sacrificed 24 hours after injection of radio-iodine. Note the black granules in the colloid, but there is no more than the background fog over the cells. $\times 400$.

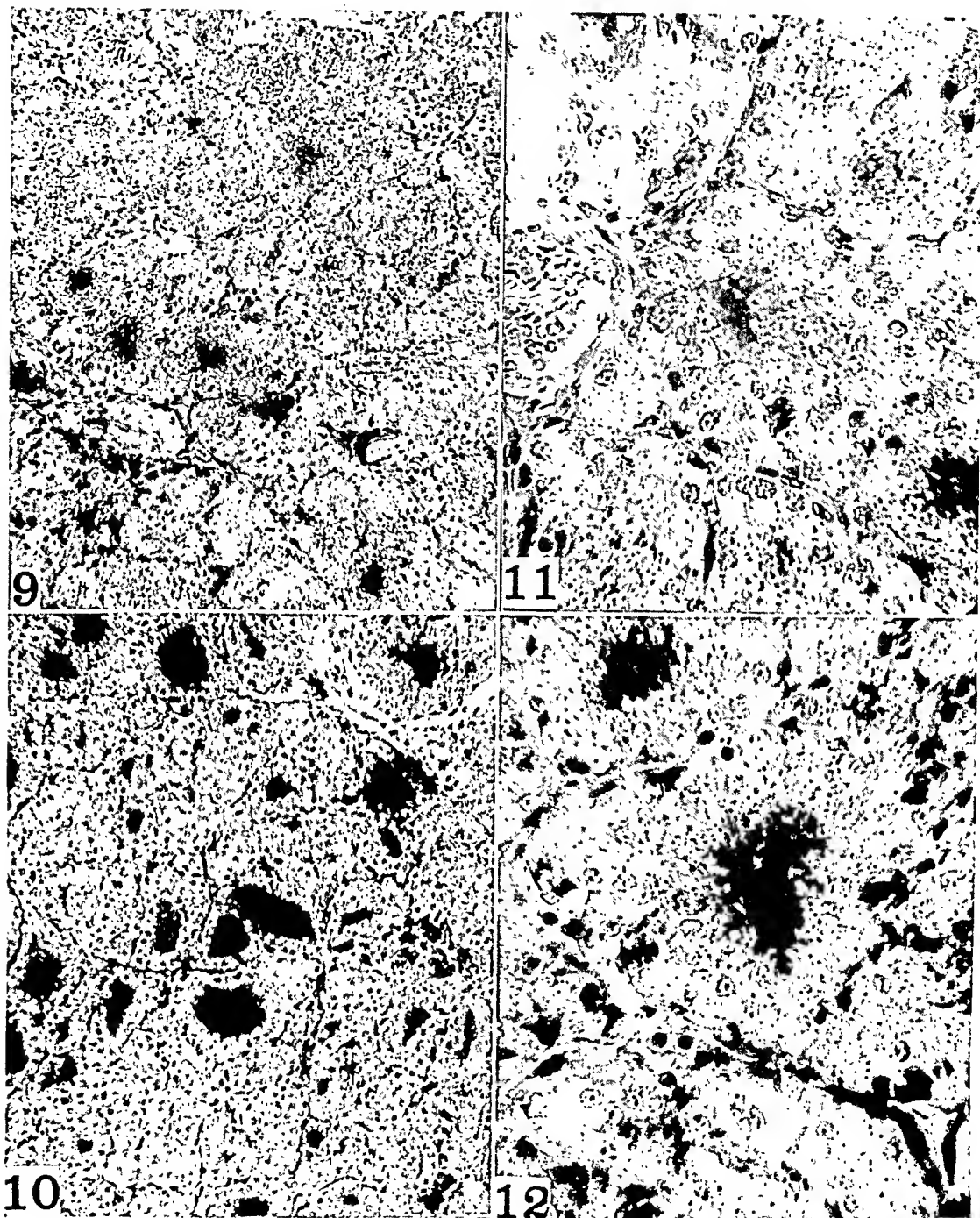


PLATE 4

H. E. stained coated autographs of thyroids of intact rats on iodine deficient diet—short exposures.

FIG. 9. The animal was sacrificed 1 hour after radio-iodine injection. The cells are large and show no reaction. Whenever the small colloid lumen happens to be in the plane of section, a reaction is apparent therein. $\times 125$.

FIG. 10. The animal was sacrificed 24 hours after radio-iodine injection. Same as above. $\times 125$.

FIG. 11. The animal was sacrificed 1 hour after radio-iodine injection. A larger magnification confirms that the cells do not contain enough radioactivity to produce a photographic image in excess of the background fog during the short exposure. $\times 400$.

FIG. 12. The animal was sacrificed 24 hours after radio-iodine injection. Same as above. $\times 400$.

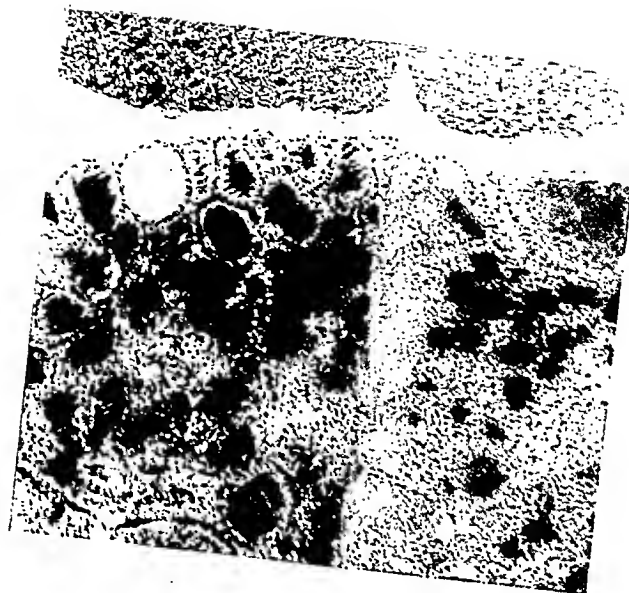


Fig. 13



Fig. 14

Fig. 13. Masson stained coated autograph of thyroid from rat given 20 micrograms of inactive iodine daily, and sacrificed 24 hours after subcutaneous injection of radio-iodine.

Most central follicles fix a considerable amount of radioactivity in their colloid as shown by the intense black reaction. These follicles stain green, but the colour is obscured by the photographic reaction.

The red-staining follicles show only a fine stippling, indicating the presence of a lesser amount of radioactivity. $\times 100$.

Fig. 14. Masson stained coated autograph of thyroid from rat on low iodine diet, and sacrificed 2 minutes after the intravenous injection of radio-iodine. Photographic granules (printed as dark green dots) are visible in the colloid of the thyroid follicles. There is no reaction in parathyroid, muscle and connective tissue. $\times 250$.

dry's finding (1922) that the Golgi apparatus, although usually apical, may be basal in some cases, and from Bensley's observation (1916) of colloid-like droplets at the base of the cells. The success of this theory was, however, not supported by more than presumptive evidence. Thus the finding of only one basal Golgi in about 500 cells in the case of Cowdry's observations (1921) was hardly indicative of colloid resorption.⁵ As for Bensley's droplets (1916), in our experience they appeared watery and not colloid-like and at any rate, no valid reason existed to link them with secretion into blood vessels. Since the present observations showed that all follicles were in the process of transforming the newly entering iodine and secreting it into the colloid, none could be said to have shifted from secretion to "reabsorption" of colloid. Therefore, no support for the reversal theories was offered by these results.

Since the rings indicative of a cellular localization of neo-thyroglobulin appeared as continuous closed rings (Fig. 1), all the cells of each follicle must have been working during the short period of time—one hour—between radio-iodine injection and autopsy of the animals. Similar observations have been made previously in 2 series of rats on a "Purina Fox Chow" diet sacrificed a half hour after radio-iodine injection (see Fig. 70 in Gross and Leblond, 1946). Since these results were obtained with a tracer dose over a rather short period of time, it could be concluded that *at all times all the cells in the ring-reacting follicles (and by inference in all follicles) were continuously active*. This result made it possible to disregard the theories assuming that only some cells were active at a time, and especially the hypothesis that the so-called "colloid cells" were responsible for the secretion of the thyroid gland.⁶

Most follicles with intra-cellular radioactivity showed a fairly even distribution of the reaction throughout their epithelium and therefore, all the cells in any given follicle must have been working at a fairly similar rate. The only exception was found in the peripheral follicles which exhibited a somewhat greater deposition of material on the side closest to the center of the gland (Fig. 1). Possibly, the lesser development of the circulation under the capsule may explain the smaller reactions in this location, since a decrease in circulation should result into a decreased supply of thyrotrophic hormone, iodide and general nutrients.

It should be emphasized that the rate of iodine incorporation was different in various parts of the gland. Thus, in general, the more central follicles made and deposited thyroglobulin more rapidly than

⁵ Giroud (1926) and Turchini (1927) have demonstrated that the inversion of the Golgi apparatus, such as described by Cowdry, was due to mechanical factors. Okkels (1931) confirmed the work of these authors and concluded from studies on normal and pathological glands that the Golgi apparatus changed in size with changes in cellular activity, but remained apical, a fact also noted by Wahlberg (1933).

⁶ Langendorff (1889); Takagi (1922); Florentin (1928); Webster (1933); Gilmore et al. (1940); Barba (1946).

did the follicles situated just under the gland capsule or at the isthmus (Figs. 1, 3, and 13; Table 2). Besides regional differences, individual follicles showed marked variations in thyroglobulin formation (Figs. 4 and 13). In general, the follicles elaborating more thyroglobulin stained green with Masson's trichrome and may be labeled basophilic, while the less active ones usually stained red and may be labeled acidophilic (Fig. 13). This result confirmed previous observations indicating a more rapid iodine turnover in the basophilic than in the acidophilic follicles (Leblond, 1943, 1944). It may be noted however, that individual differences were less pronounced in the follicles of the stimulated glands. At any rate, each follicle had individual characteristics which at least in certain locations, such as the periphery of the gland or the isthmus, appeared definitely fixed. From these various observations, it seemed likely that each follicle maintained its own rate of formation of thyroglobulin.

So far, the excretion of the hormone out of the follicle has not been considered. One of the prevalent opinions on the subject has been that the thyroid cell secreted directly into the circulation, while the colloid lumen functioned as storage space (Bensley, 1916; Bargmann, 1939). Wegelin (1926) considered this possibility as arising only in the case of stimulated glands. However, the finding that the radio-iodine was fixed in large amounts by the stimulated thyroid gland within 15 minutes after injection (Table 4), while no radioactivity above the level of experimental error could be detected in the organic fraction of the blood, was not in favor of a direct secretion into the circulation. On the other hand, it has never been possible to detect the presence of thyroglobulin itself in the blood plasma (Lerman, 1940). It was therefore concluded that the thyroid cell was secreting only in the direction of the colloid.

Whatever the rate of entry of organic iodine into the colloid lumen, maintenance of the steady state made it imperative that it be equal to the iodine output. Furthermore, since all follicles were continuously elaborating thyroglobulin, the release of the iodinated hormone into the circulation must have taken place while thyroglobulin was being made and secreted into the colloid. An explanation of excretion fitting these requirements was afforded by the hypothesis of de Robertis (1941b; Dziemian, 1943; de Robertis and Nowinski, 1946) assuming a rapid breakdown of thyroglobulin by enzymatic proteolysis in the colloid. In agreement with this theory, a substance with thyroxine-like properties, possibly thyroxine itself (Leblond and Gross, 1948) was found in thyroid tissue resulting probably from the proteolysis of thyroglobulin. In view of their small size such fragments should diffuse out of the follicle more easily than the large molecule of thyroglobulin. Indeed, thyroxine-like substances have been recovered from the plasma by Trevorrow (1939), Taurog and Chaikoff (1947) and Leblond and Gross (1948).

It may be helpful for the understanding of the follicle function to remember its entodermal origin and to venture a comparison with the

intestinal epithelium, which secretes proteins (enzymes) into the lumen in a direction consistent with the histological polarity of its cells, and at the same time resorbs from the lumen smaller molecules resulting from digestion (amino acids, . . .). Similarly, the thyroid epithelium secretes a protein (thyroglobulin) into the follicle lumen and at the same time resorbs smaller molecules probably thyroxine itself.

SUMMARY

The entry of radioactive iodine into the thyroid was observed by means of the autographic technique in the thyroids of animals kept under the following conditions: a—Rats on a low iodine intake, b—Rats receiving about 22 μ gm. of iodine daily, c—Hypophysectomized rats on a low iodine intake and d—Guinea pigs receiving thyrotrophic hormone. The autographic reactions were shown to be due to radio-iodine present as thyroglobulin.

In the animals receiving 22 μ gm. of non-radioactive iodine daily, the radioactivity is mostly present in the epithelium at 1 hour (Figs. 1, 3, and 5), and in the colloid at 24 hours after injection (Figs. 2, 4 and 6). In the iodine-deficient group, the deposition of radio-iodine into the colloid was much accelerated, since it was found there as early as 2 minutes after injection (Fig. 14). A rapid deposition is also found in the thyrotrophin-treated animals. In the hypophysectomized group, the deposition of radioactivity into the colloid is depressed, since the radioactive material is still present in the cells at 24 hours after injection (Figs. 7–8). These results indicate that thyroglobulin is formed in the cells and is released into the colloid, this sequence being most rapid in active thyroids and slow after hypophysectomy.

The secretion polarity of the thyroid cell in the direction of the colloid, suggested by the above data, is further demonstrated by the presence of a considerable amount of radio-iodine in the colloid at 2, 7 and 15 minutes after injection, although no significant amount of protein bound iodine can be detected in the blood at that time.

The presence of radioactivity in all follicles as early as 1 hour and in one case 2 minutes after radio-iodine injection (Fig. 14) shows that all follicles in any gland are active at all times in fixing iodine. However, the rate of iodine fixation by the various follicles is variable (Fig. 13); thus, in the rat less active follicles are located under the capsule and in the isthmus of the gland.

From the data presented, it seems likely that circulating iodide is continuously bound to protein (i.e., thyroglobulin) in the cytoplasm of the thyroid cell. This thyroglobulin is continuously deposited in the colloid of the thyroid follicle. Excretion of the thyroid hormone itself probably results from the proteolytic breakdown of thyroglobulin in the colloid and the diffusion of the resulting fragments through the epithelial wall of the follicle.

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MECHANISMS OF DESOXYCORTICOSTERONE ACTION: EFFECTS OF LIVER PASSAGE

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THE EVOCATION of hypertensive cardiovascular renal disease in experimental animals through desoxycorticosterone administration suggests that essential hypertension and its sequellae may be the result of increased activity by endogenous salt-retaining corticosteroids. Such a possibility does not necessarily imply greater production of cortical substances but might represent a defect in removal.

Detoxification of gonadal steroids is accomplished in the liver (Engel, 1944; Kochakian, Haskins and Bruce, 1944; Krichesky, Benjamin and Slater, 1943). The experimental data relative to the adrenal steroids are somewhat equivocal. The inactivity of desoxycorticosterone when orally administered (Kuizenga, Nelson and Cartland, 1940), its chemical similarity to progesterone and the identity of at least one of the detoxification products of both substances (Hoffman, Kazmin and Browne, 1943) suggest a common metabolic pathway through the liver. This supposition is supported by the decreased growth and survival rates of adrenalectomized animals when desoxycorticosterone pellets are implanted in the mesentery or spleen rather than in the subcutaneous tissues (Eversole and Gaunt, 1943). Whole cortical extract, in contrast, is active when given by mouth and relieves the symptoms of Addison's Disease, although the dose must be increased three- or four-fold (Thorn, Emerson, and Eisenberg, 1938). Furthermore adrenal cortical transplants show evidences of biologic activity when grown in the mesentery of the host animal (Eversole, Eddman and Gaunt, 1940).

The sum of these results would seem to indicate that the passage of corticosteroids through the liver results in detoxification but not to the extent of preventing the maintenance of life. Survival and growth, however, may be accomplished by the escape of small quantities of active steroids into the circulation, while large amounts would not enhance these effects proportionately. Consequently, it was considered desirable to re-evaluate the role of the liver in the removal of desoxycorticosterone by measuring the effects of hepatic passage on those actions seen at pathologic dosage levels, the intensities of which are more or less proportional to the quantity of circulating drug.

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Continuous administration of high desoxycorticosterone dosages through the use of subcutaneous implants is followed by a progressive elevation of fluid intake, complete in approximately ten days (Green and Glover, 1948; Green, Coleman and McCabe, in press). The new level of intake is related to that of controls of the same weight by a ratio fixed by the drug dosage and the degree of supplementary sodium chloride administration (Green, Coleman and McCabe, in press).

Hypertension develops in such implanted animals subsequent to the attainment of the new fluid intake level (Green and Glover, 1948; Green, 1948). When single 20 mg. pellets are used, the blood pressure climbs slowly and reaches a maximum in approximately three months. The hypertension is attended by cardiac and renal hypertrophy (Green, Coleman and McCabe, in press). Some animals die during the period of most intense blood pressure elevation. All of these manifestations are intensified if isotonic sodium chloride solution is substituted for drinking water (Green, Coleman, and McCabe, in press).

The degree to which these toxic actions are influenced by the passage of desoxycorticosterone through the liver is the subject of this report.

PROCEDURE

Single 20 mg. DCA pellets were implanted in the spleen of 5 half-grown rats of the Sprague-Dawley strain. Five control animals underwent a sham operation. Single 20 mg. pellets also were implanted in 10 animals subcutaneously. Eight normal animals served as controls for the latter group. All animals were kept in separate cages and were fed Purina Laboratory chow ad libitum. Isotonic (0.86%) saline solution was substituted for drinking water. Fluid intake was measured daily and weight weekly. At the conclusion of the 12-week period, previously shown to be the time required for the development of maximum blood pressure elevation under the circumstances described, multiple blood pressure readings were made on all surviving animals. The animals were then sacrificed and organ weights were determined.

RESULTS

The fluid intake of animals subcutaneously implanted with pellets showed a prompt rise, maximal between the first and second weeks.

TABLE 1. THE EFFECTS OF INTRASPLENIC IMPLANTATION OF DCA PELLETS ON THE BLOOD PRESSURE OF RATS AS COMPARED WITH SUBCUTANEOUS IMPLANTATION.

Drinking Fluid (0.86% NaCl)	No. of Animals	Average Blood Pressure
One 20 mg. DCA Implant Intrasplenically	4	128
One 20 mg. DCA Implant Subcutaneously	5	180
Controls:	12	114

This new level of intake, approximately twice that of the controls, was maintained throughout the experiment (Fig. 1).

By comparison, the change in the fluid consumption of animals implanted intrasplenically was minor. The maximum level of intake

RATIO OF INTAKE, TEST GROUP,
TO INTAKE, CONTROL GROUP

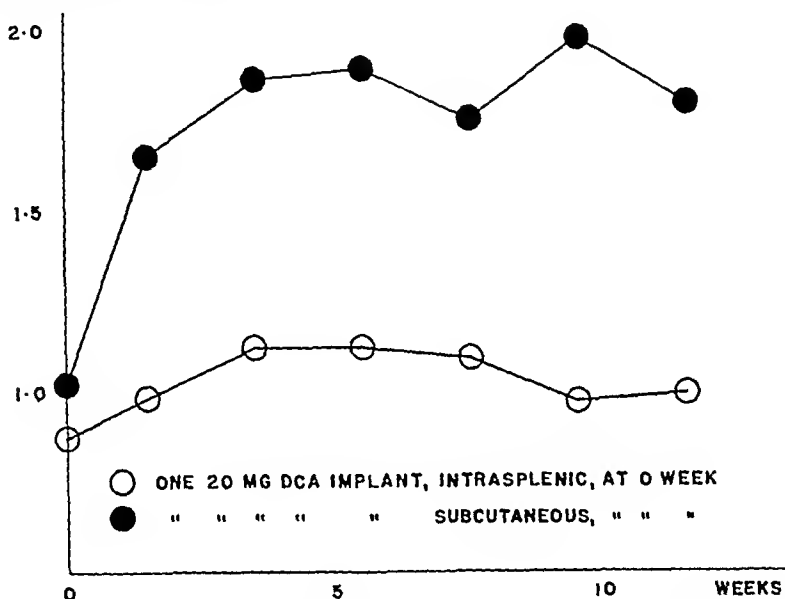


FIG. 1. The effect of intrasplenic implantation of DCA pellets on the fluid intake of rats, as compared with subcutaneous implantation. (Drinking Fluid: 0.86% NaCl).

differed from that of the sham operated group by approximately 10 per cent and was not sustained.

The average maximum blood pressure of the animals subcutaneously implanted was 180 mm. mercury as compared with 128 mm. for those implanted intrasplenically (Table 1). The average of all control blood pressures was 114 mm. mercury.

Four of the 5 animals implanted intrasplenically survived the

TABLE 2. PERCENTAGE HEART AND KIDNEY WEIGHTS IN RATS FOLLOWING INTRASPLENIC IMPLANTATION OF DCA PELLETS AS COMPARED WITH SUBCUTANEOUS IMPLANTATION.

Drinking Fluid (0.86% NaCl)	Percentage Heart Weight	Percentage Kidney Weight
One 20 mg. DCA Implant Intrasplenically	0.36	1.01
One 20 mg. DCA Implant Subcutaneously	0.92	1.29
Controls:	0.33	0.90

entire period of study. One of this group died subsequent to the operative procedure, as did one of the sham operated animals. In contrast, only one half of the subcutaneously implanted group survived the 12-week period. The others died at intervals during the second and the third months following implantation.

The ratios of heart and kidney weights to body weight of the animals implanted intrasplenically did not differ materially from those of controls (Table 2). When pellets were placed under the skin, however, these organs became uniformly hypertrophied. The percentage weights of animals in this group which died spontaneously seemed to be exaggerated in some instances by the weight loss which preceded their death.

Inspection of the implantation sites at autopsy indicated that the intrasplenic pellets had been absorbed somewhat more completely than those placed under the skin.

SUMMARY AND CONCLUSIONS

The marked alterations in fluid exchange, blood pressure, organ weight and survival which followed the subcutaneous implantation of 20 mg. DCA pellets in growing rats were reduced to minor values when pellets of the same size were implanted intrasplenically. It was concluded that the rat liver possessed a capacity for detoxifying desoxycorticosterone in quantities potentially capable of producing hypertensive disease and its organic sequelae. No evidence was secured to indicate effective use of this capacity when the drug was introduced outside of the portal circulation. The failure of the liver to protect the animal from subcutaneous DCA pellets probably was attributable to the relatively small fraction of the systemic blood flow which passed through the hepatic and portal vessels during any one circulation.

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ANTERIOR PITUITARY GROWTH PREPARATIONS: THE RELATIONSHIP BETWEEN NITROGEN STORAGE AND OTHER CRITERIA OF ACTIVITY¹

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RESULTS of early experiments with anterior pituitary growth preparations led to a series of well correlated findings. In spite of a large increase in the metabolic rate, nitrogen storage was produced with the food intake constant. The increased caloric requirement was met by oxidation of fat (Gaebler, 1933). Storage of nitrogen was accompanied by increase in weight, and by diminished excretion of inorganic sulfate and phosphate (Gaebler and Price, 1937), suggesting synthesis of proteins, of other compounds containing nitrogen and sulfur, or of phosphorus compounds. Moreover, results in the absence of the thyroid, parathyroids, pancreas, and adrenals (Gaebler and Robinson, 1942) indicated that part of the effect on nitrogen storage might well be produced in the tissues themselves. On the basis of this experience it appeared to us that growth preparations, as distinct from purified growth hormone, might continue to be of value in studying the mechanism by which nitrogen storage is produced.

In such experiments, nitrogen storage would seem to be the logical test for activity of the preparations used, unless all assays yielded concordant results, whether based on weight, nitrogen storage, or urinary phosphate in dogs, the gain of weight in plateaued rats (Marx, Simpson, and Evans, 1942), or the epiphyseal disc response in hypophysectomized rats (Evans, Simpson, Marx, and Kibrick, 1943). In this event the determination of urinary phosphate in dogs receiving growth preparations would be the simplest assay. Gordan, Bennett, Li, and Evans (1948) obtained good correlation between the effects of purified growth hormone on weight and nitrogen storage in rats. In recent work on dogs, with assayed growth preparations supplied by several reliable firms,² we were at first unable to obtain correlation

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between any of the five assays mentioned above. In the end, however, the value and limitations of nitrogen storage, gain in weight, and urinary phosphate as criteria of activity became fairly clear.

DIETS AND METHODS

Diet 1 consisted of beef heart trimmed free of fat, cracker meal, and bone meal. Constancy in a given experiment, and adaptability to caloric requirements of different animals, was provided for as follows: 7000 gm. of trimmed beef heart was ground, mixed with 420 gm. of bone meal, and put up in 193 gm. packs which were frozen until required. A mixture of 1630 gm. of ground trimmed beef heart and 3080 gm. of cracker meal, dried to 85 per cent of its original weight, was refrigerated in air tight cans. Each animal received daily one pack of beef heart and an amount of the mixture suited to its caloric needs. Diet 6 consisted of casein, 36 per cent; cracker meal, 36 per cent; corn oil, 20 per cent; powdered brewer's yeast, 4 per cent; and Phillips-Hart (1935) salt mixture, 4 per cent. All animals received 3 drops of haliver oil daily.

Analytical methods were the same as in earlier studies already cited. Complete nitrogen balances were done in some experiments, but nitrogen storage was usually calculated from the urine nitrogen alone, since earlier work had justified this procedure. The average of 5 or more days preceding injection was taken as the control level of nitrogen output. Since loss of nitrogen was frequently observed during the first 24 hour period following injections, irrespective of the preparation tested, this day was excluded from calculations. The period for which storage was computed ended when the urine nitrogen returned to or exceeded the control level, and was thus of variable length. Food intake was constant throughout control and experimental periods of each test.

RESULTS

The first 6 experiments shown in Table 1, hitherto unpublished, were carried out many years ago with an early type of preparation and diet 1. The amount of nitrogen stored varied greatly in individual experiments, although the diet, growth preparation, and dosage were the same throughout. However, the average amount of nitrogen stored per kilo is nearly identical for dogs 1 and 4,—0.502 and 0.498 gm. This calculation was suggested by the statement of Kochakian (1946) that the maximal rate of nitrogen storage produced by steroid hormones is directly proportional to weight in dogs. In the last 2 experiments in Table 1, the latest type of preparation and diet 6 were employed. Storage of nitrogen is again in the vicinity of 0.5 gm. per kilo. We therefore consider a preparation highly active if, when a 200 mg. dose is injected subcutaneously into dogs receiving a complete diet containing 10 gm. or more of protein nitrogen per day, it produces nitrogen storage of 0.5 gm. per kg. This may well be a maximal response. Before attempting to convert the procedure into an assay by using successively smaller amounts of the preparation, it seemed judicious to examine the relationship between nitrogen storage and other effects.

In pair fed rats receiving unpurified growth preparations (Lee and Shaffer, 1934) the excess weight which was added contained 4.8 per cent of nitrogen, as determined by actual analysis. Correlation of observed and calculated weight increase in rats receiving purified growth hormone (Gordan, Bennett, Li, and Evans, 1948) was obtained by assuming 16 per cent of nitrogen in protein and 20 per cent of protein in tissue, thus 3.2 per cent of nitrogen in the added weight. In our experiments on dogs receiving large single injections of growth preparations the amount of nitrogen stored per 100 gm. of gain in weight is much less (Table 1, second last column). In other words, gain in weight was 38 to 71 per cent greater than would be predicted on the basis of 3.2 per cent of nitrogen. Thus nitrogen stored per 100 gm.

TABLE 1. STORAGE OF NITROGEN AND INCREASE IN WEIGHT FOLLOWING SINGLE LARGE INJECTIONS OF ANTERIOR PITUITARY GROWTH PREPARATIONS

Dog No.	Weight kg.	Diet	Preparation	Date	Nitrogen Stored			Weight gain gm.
					Total gm.	Grams per kg.	Grams per 100 gm. gain in wt.	
1	21.2	1	095052A	5-12-32	14.38			453
1	20.8	1	095052A	6-4-32	9.37			566
1	23.5	1	095052A	11-17-32	9.08			398
Average	21.8				10.94	.502	2.32	472
4	22.3	1	095052A	6-21-32	7.40			453
4	24.4	1	095052A	10-19-32	14.63			680
4	23.9	1	095052A	1-26-33	13.06			740
Average	23.5				11.70	.498	1.88	624
44	16.2	6	099783		7.54	.465	2.04	370
42	16.45	6	099816		9.94	.604	1.74	570
Average						.535	1.89	470

All injections were given subcutaneously. In each of the first 6 experiments, dogs 1 and 4 received 50 cc. of the same preparation of Antuitrin G. In each of the last 2 experiments, dogs 44 and 42 received 200 mg. of preparations also made by Parke, Davis and Co., but according to the recent procedure of Fishman, Wilhelmi, and Russell (1947). These preparations assayed 1485 and 1361 R.U. respectively.

of gain in weight has been found to vary from 3.2 to 4.8 gm. in the same species, and from less than 2 gm. to 4.8 gm. in different species, under varied experimental conditions which can be standardized.

Difficulties which came to light in the following experiments are perhaps more serious. That anterior pituitary preparations may contain substances producing nitrogen loss is shown in Table 2. The preparations injected were made as nearly as possible in the same way as the Antuitrin G used in experiments shown in Table 1. Although diet 6 had proven satisfactory (Table 1), we assured ourselves that supplementing it with 50 mg. of riboflavin daily made no difference, for diet 1 contained a great deal of beef heart, which is very rich in this factor. In Table 3 a comparison of preparations from two firms is

presented. Preparation G-731, supplied by Parke, Davis and Company, and found by them to assay 1150 R.U. per gm., was completely ineffective in dog 41 when given in 100 and 200 mg. doses. In dog 42, some nitrogen was stored, but there was loss in weight. Referring again to Table 1, it will be seen that maximal nitrogen storage was produced by preparations only 18 to 29 per cent more potent as as-

TABLE 2. SHOWING THAT ANTERIOR PITUITARY PREPARATIONS PREPARED IN THE SAME MANNER AS HIGHLY POTENT GROWTH PREPARATIONS PRODUCED NITROGEN LOSS

Preparation	Source	Dog	Weight kg.	Diet	Nitrogen Stored	
					Total gm.	Gram per kg.
099506	hog	41	15.4	6	-5.86	-0.380
099507	hog	41	15.2	6	-5.30	-0.349
099507	hog	42	16.1	6	0.86	0.053
099529	beef	42	16.0	6	2.50	0.156
099507	hog	41	15.0	6R	-8.80	-0.587
099529	beef	41	14.5	6R	0.54	0.037
099506	hog	42	15.8	6R	-1.64	-0.104
099529	beef	42	15.9	6R	-2.45	-0.154

Diet 6R consisted of diet 6 plus 50 mg. of riboflavin daily.

In each experiment, 40 cc. of anterior pituitary extract equivalent to 20 gm. of fresh anterior lobes was injected.

TABLE 3. COMPARISON OF NITROGEN STORAGE PRODUCED BY TWO PREPARATIONS OF SIMILAR POTENCY AS JUDGED BY OTHER METHODS OF ASSAY

Dog	Weight kg.	Diet	Preparation	Dose mg.	Nitrogen Total gm.	Stored Grams per kg.	Weight change kg.
41	15.7	6	G-731	100	0.61	0.039	0.0
41	15.7	6	G-731	200	0.39	0.025	-0.1
42	15.4	1	G-731	200	3.42	0.222	-0.2
42	15.0	1	R-95	195	9.71	0.648	0.0

Preparation G-731 was lyophilized extract of hog anterior lobes and assayed 1150 R.U. per gm. After the above experiments, on Aug. 15, 1947, it assayed 41 epiphyseal disc units per mg. Preparation R-95 assayed 45 epiphyseal disc units per mg., $\pm 28\%$, some time before, and 30 epiphyseal disc units, $\pm 19\%$, on Aug. 15, 1947, after the above experiments.

sayed in the same laboratory. Since maximal nitrogen storage was produced with preparation R-95 from Armour and Company, permission was secured to send preparation G-731 to that firm for comparison with R-95 in the same laboratory. As stated in the footnote to Table 3, preparation G-731 was, in epiphyseal disc units, at least as potent as R-95. It should also be noted that the large nitrogen storage obtained with the latter preparation was unaccompanied by change in weight.

In Table 4 are recorded the results of two injections of an unsatisfactory growth preparation. Nitrogen was lost in each experiment, yet urinary phosphate fell quite definitely in both. The same anomaly was observed in 6 other experiments with various anterior pituitary preparations. In earlier experiments (Gaebler and Price, 1937), which

included phosphorus balances, there was a general parallelism between nitrogen and phosphate output in the urine. It was noted, however, that while the loss of nitrogen so often seen on the day of injection was accompanied by increased output of inorganic sulfate there was a fall in phosphate excretion on this day. The general parallelism was therefore not accepted as evidence for synthesis of nucleo-protein. It may be mentioned here that deposition of calcium phosphate, though part of the growth process, is not necessarily synchronous with nitrogen retention.

The explanation that growth preparations may contain substances

TABLE 4. DIMINISHED URINARY PHOSPHATE EXCRETION AFTER INJECTION OF ANTERIOR PITUITARY PREPARATIONS WHICH CAUSED NITROGEN LOSS

Day of experiment	Water intake, cc. per day	Urine vol., cc. per day	Weight kg.	Total urine nitrogen, gm.	Total urine inorganic phosphate, gm. as P.
1	1160	765	15.88	8.53	
2	1260	780	15.88	9.23	
3	1235	620	15.76	9.12	0.85
4	1365	680	15.88	8.95	0.81
5*	1620	1030	15.76	10.91	0.82
6	2690	1940	15.76	10.60	0.39
7	2680	1480	15.65	8.92	0.49
8	1745	1100	15.65	8.46	0.54
9	1285	1040	15.76	9.55	0.69
10-12	1210	610	15.92	9.15	0.76
13-15	1030	455	15.92	8.15	0.63
16**	1940	910	16.33	10.20	0.59
17	1400	1365	15.99	10.42	0.48
18	1470	980	15.88	9.02	0.61
19	1015	600	15.88	8.96	0.76
20	1145	610	15.99	8.56	0.74
21	950	480	15.99	9.16	0.70

* 20 cc. of aqueous alkaline hog anterior pituitary extract No. 099506 injected subcutaneously. The injection was repeated on the 6th day.

** 20 cc. of aqueous alkaline beef anterior pituitary extract No. 099529 injected subcutaneously. The injection was repeated on the 17th day. The animal received diet 6R (see footnote, Table 2) during both experiments.

which cause nitrogen loss when large single injections are given seems to cover most of the irregularities which we observed, and is supported by the data in Table 2. Since the newer preparations with which we had difficulties were much more highly purified than older preparations, we considered the alternative possibility that both nitrogen storage and gain in weight are effects of synergistic substances which can become separated. However, production of well correlated nitrogen storage and gain in weight with purified growth hormone (Gordan, Bennett, Li, and Evans, 1948) limits one at once to combined effects of growth hormone and impurities which are not chemically or biologically demonstrable. Moreover, experiments with mixtures of preparations yielded the results shown in Table 5. The thyrotropic preparation T-7210 slightly accentuated nitrogen storage when given

with G-731, and virtually abolished it when given with R-95. The pituitary gonadotrophin preparation FWP-234 produced, in one experiment, an effect very similar to that of gonadotrophin from pregnancy urine (Gaebler, 1935). Nitrogen output fell only after several days and remained below the control level for some time. In the other experiment with FWP-234 there was nitrogen loss. The potent prolactin preparation FWP-12 caused nitrogen loss in both experiments, although, in agreement with others, we have previously reported marked nitrogen storage after injection of prolactin preparations.

TABLE 5. EFFECTS OF MIXTURES OF ANTERIOR PITUITARY PREPARATIONS ON WEIGHT AND NITROGEN STORAGE

Dog	Weight	Diet	Preparations	Doses mg.	Nitrogen Stored Total gm.	Grams per kg.	Weight change kg.
42	15.4	1	G-731	200	3.42	0.222	-0.20
42	14.5	1	G-731 + T-7210	200 + 100	5.28	0.365	-0.16
42	15.0	1	R-95	195	9.71	0.648	0.00
42	14.4	1	R-95 + T-7210	200 + 100	1.26	0.087	-0.11
42	15.7	6	FWP-234	200	8.41	0.535	-0.12
42	16.2	6	FWP-12	200	-4.79	-0.296	0.00
42	16.4	6	FWP-234 + FWP-12	100 + 100	-4.62	-0.282	0.00
44	15.7	6	FWP-234	200	-4.38	-0.279	-0.17
44	15.8	6	FWP-12	200	-1.09	-0.069	-0.12
44	16.1	6	FWP-234 + FWP-12	100 + 100	-1.41	-0.087	0.00

T-7210 was a thyrotropic preparation which produced loss of both nitrogen and weight in an experiment on dog 40. G-731 and R-95 were growth preparations already described in the footnote of Table 3. FWP-234 was an Armour sheep anterior pituitary gonadotrophic preparation which the firm's assay showed to contain 159 Collip U. per mg. Other factors reported present were: thyrotropin, 0.4 chick unit per mg.; growth hormone, 0.2 epiphyseal disc units; adrenocorticotropin, 0.2% of Armour's standard; prolactin, none. FWP-12 was an Armour prolactin preparation which assayed 72 i.u. per mg. This preparation was not tested for contaminating hormones, but preparations similar to FWP-12 contained adrenocorticotrophin equivalent to 1.5% of Armour standard. Presence of appreciable amounts of gonadotrophic, thyrotropic, or growth hormone was considered unlikely.

Combination of gonadotrophin and prolactin preparations produced nitrogen loss in both experiments. In none of these tests was there notable change in weight. Weights of these large animals are subject to spontaneous fluctuation of about 0.11 kg. under our conditions. The effects of mixtures of anterior pituitary preparations on nitrogen storage appeared to be more erratic than those of single preparations, as might be expected if the irregularities are due to substances causing nitrogen loss.

SUMMARY

Growth preparations may be considered satisfactory if, when injected subcutaneously in a large single dose of 200 mg., they produce nitrogen storage of 0.5 gm. per kg. in dogs maintained on a complete diet high in protein. The gain in weight following such injections is greater than would be expected, nitrogen stored per 100 gm. of gain in weight being about 2 per cent. There is thus evidence for water

retention in excess of requirements for new tissue. Failure of correlation between nitrogen storage and other criteria of activity appears to be due to presence, in some growth preparations, of substances causing nitrogen loss, rather than to absence of any substance acting synergistically with the growth hormone to produce nitrogen storage. Phosphate output in the urine often declines even if the preparation or mixture of preparations injected causes nitrogen loss.

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THE CORRELATION OF THE CIRCULATING POLY-MORPHONUCLEAR LEUCOCYTES (NEUTROPHILES) WITH THE ADRENAL ASCORBIC ACID IN THE RAT

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CLINICAL and experimental animal data reported in the past few years have indicated that there probably is a functional relationship between the activity of the adrenal cortex and the hematologic picture of individuals. Alterations in the normal white blood cell composition were reported in endocrinopathies of the adrenal cortex and the pituitary-adrenal cortex system (Balze, Reifenstein and Albright, 1946). The induction of a change from the normal white blood cell picture following ACTH in patients with adequate adrenal-cortical function has been reported by Forsham, Thorn, Prunty and Hills (1948). The hematologic changes observed in humans following alteration of adrenal-cortical function as a result of pathology or experimentally were predictable from the investigations on lower mammals by Dougherty and White (1944, 1947). The latter investigators have shown that increased activity of the adrenal cortex, particularly secretions of the 11-oxysteroid type, results in a lymphopenia specifically and a non-specific rise in the polymorphonuclear leucocytes. Selye (1940) and Reinhardt, Aron and Li (1944) had shown that injections of ACTH in rats induced a lymphopenia and a neutrophilia. Selye (1937, 1946) had reported that non-specific agents acting via the pituitary-adrenal cortical mechanism induced a lymphopenia and a polymorpho-leucocytosis.

In both the clinical and animal studies the emphasis had been placed on the relation of the adrenal cortical activity to the circulating number of lymphocytes. However, a polymorpho-leucocytosis has consistently been shown to occur with an increase in adrenal-cortical activity when these have been reported. Only recently has it been pointed out that one type of granular leucocyte, the eosinophiles, apparently vary with and are possibly indicative of adrenal-cortical activity (Forsham, Thorn, Prunty and Hills, 1948). The latter investigators also reported a neutrophilia after ACTH injections in humans but claimed this to be a non-specific effect since it occurs to some extent after ACTH in Addisonian patients.

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It has been shown that a decrease in the adrenal ascorbic acid or cholesterol content of fresh adrenal glands may be used as an indication of adrenal-cortical activity (Sayers, Sayers, Fry, White and Long, 1944; Sayers, Sayers, Lewis and Long, 1944; Sayers, Sayers, Liang and Long, 1946; Sayers and Sayers, 1946). Statistical evidence that the depletion of the ascorbic acid content of the adrenal can be used as a measure of the potency of ACTH preparations and of the activity of the adrenal cortex has recently been reported by Sayers, Sayers and Woodbury (1948). Long (1947) has presented material which indicated that the pattern of depletion (per cent change plotted against time) of the adrenal ascorbic acid was similar to the pattern of change in the circulating number of lymphocytes after ACTH injections in the rat. However, in the same paper Long quoted Dr. Oesterling's unpublished data which showed that in the scorbutic guinea pig the decrease in circulating lymphocytes was matched by a decrease in the adrenal cholesterol but no change in the already low adrenal ascorbic acid. The polymorphonuclear leucocytes were not discussed.

In view of the changes which were reported in both the lymphocyte and neutrophile picture following adrenal-cortical stimulation or adrenal-cortical endocrinopathy it seemed that a study of the relationship of these white blood cells with the adrenal ascorbic acid might elucidate the problem. Such a study appeared feasible since adrenaline had been shown to actively influence the number of circulating lymphocytes via adrenal-cortical stimulation (Long, 1947) and it had also been shown that a lymphopenia could be induced in urethanized rats which was not mediated through the adrenal gland (Dury and Robin, 1948). It was also reported that adrenaline injections in urethanized rats induced a prolonged neutrophilia. These points suggested that both adrenaline and urethane could be used as tools to actively alter the composition of the white blood cell picture and possibly yield information concerning the physiological relationship, if any, between the adrenal ascorbic acid and the circulating lymphocytes or polymorphonuclear leucocytes.

This paper will present evidence of the statistically significant correlation of the adrenal ascorbic acid with the polymorphonuclear leucocytes and the alteration of this relationship with certain drugs.

MATERIALS AND METHODS

Adult male rats of the Sprague-Dawley strain weighing 200-250 grams were used in these studies. The rats were fed Purina Chow and tap water ad lib., and lettuce was given once weekly. Total and differential leucocyte determinations were done by standard procedures on the blood obtained from the tails of the rats. The differential leucocyte values were computed after counting at least one hundred white cells on blood smears stained with Wright's blood stain. The adrenals were removed from the rats immediately after the blood was drawn for the cell counts, cleaned of fat and weighed on a

Roller-Smith precision balance to the nearest tenth of a milligram. The adrenal ascorbic acid content was determined by the method of Roe and Kuether (1943). The data were statistically evaluated by procedures suggested by Snedecor (1946). Evipal was used for anesthetic purposes.

The leucocyte picture and the adrenal ascorbic acid were studied in five different groups of rats as follows:

Group *a*. Sixteen normal intact rats in our colony were elected at random for the determinations of their adrenal ascorbic acid and leucocyte composition. These were considered the control group for this study.

Group *b*. Leucocyte counts were made before and three hours after a course of adrenaline injections in this series of fourteen rats. The adrenaline used was a 0.02% solution made up fresh for each set of injections. The rats were injected according to a procedure recommended by Long and Fry (1945) to attain adrenal-cortical stimulation. The adrenaline was injected subcutaneously at four successive hourly intervals at a dose level of 0.02 mg. per 100 gm. of body weight. Three hours after the last adrenaline injection the rats were lightly anesthetized with Evipal, blood was drawn from the tails for the leucocyte determinations and differentials, and the adrenals removed for ascorbic acid analysis.

Group *c*. All procedures were the same as above with the exception that the period between the last adrenaline injection and the final leucocyte and the ascorbic acid determinations was a total of twenty hours.

Group *d*. The rats comprising this group were given intraperitoneal injections of a 10% urethane solution every other day for a total of eight injections at a dose level of 750 mg. per kg. of body weight. The day after the last urethane injection determinations were made of the leucocyte counts, differentials, and the adrenal ascorbic acid.

Group *e*. The procedures in the rats comprising this series were exactly the same as the above group with the exception that on the day after the eighth urethane injection these rats were also given the course of adrenaline injections. Leucocyte counts, differentials, and adrenal ascorbic acid determinations were done three hours after the last adrenaline injection.

The three hour and the twenty hour periods after the last adrenaline injection was chosen as an end point on the basis of data in the literature. It had been shown that the lymphopenia and the polymorpho-leucocytosis were at a maximum approximately three hours after ACTH injections in rats; and that by approximately twenty hours after the ACTH injection these cell types were again at approximately their normal circulating number (Dougherty and White, 1944, 1947). Forsham, Thorn, Prunty and Hills (1948) had also shown that the maximum fall in the circulating lymphocytes and eosinophiles, and the maximum rise in the circulating neutrophiles occurred approximately four hours after ACTH injections in humans. The adrenal ascorbic acid had been shown to be maximally depressed one to three hours after ACTH injections and gradually approximates the normal value twenty hours after the injection (Sayers, Sayers, Liang and Long, 1946; Long, 1947).

RESULTS

The average absolute number of lymphocytes, polymorphonuclear leucocytes (in the rat these are practically all neutrophils), the total white blood cell count per c.mm. of blood and the average adrenal

TABLE 1. THE LEUCOCYTIC PICTURE AND THE ADRENAL ASCORBIC ACID CONTENT OF NORMAL INTACT, ADRENALINE TREATED, AND URETHANIZED RATS

Animal used	Lymphocytes absolute number per c.mm.	"p"*	Polymorpho- leucocytes absolute number per c.mm.	"p"	W B C per c.mm.	"p"	Adrenal ascorbic acid mg./100 gm. fresh gland	"p"
NORMAL:								
a. Controls (10)	10,978 ± 964**		5,009 ± 689		22,138 ± 1,189		300 ± 10.35	
b. 3 hours after*** adrenaline (14)	4,984 ± 422	(a, b) <0.01	19,454 ± 2,072	<0.01	24,440 ± 2,288	>0.3	320 ± 10.0	<0.01
c. 20 hours after adrenaline (14)	10,608 ± 1,385	(b, c) <0.01 (a, c) <0.01	6,044 ± 1,220	>0.2	17,450 ± 2,164	>0.02 >0.05	379 ± 20.70	>0.02 >0.5
URETHANIZED:								
d. Controls (8)	7,664 ± 810	(a, d) <0.01	0,166 ± 1,213	>0.4	13,831 ± 1,094	<0.01	383 ± 28.84	>0.5
e. 3 hours after adrenaline (9)	2,808 ± 418	(d, e) <0.01 (b, c) <0.01	15,510 ± 1,787	<0.01 >0.1	18,244 ± 1,912	>0.1 <0.05	403 ± 32.01	>0.02

Number in () is the number of rats in the experiment.

* "p" is the value of the Difference between the Means occurring by chance alone.

** Mean ± S.E. of the Mean

*** Adrenaline given i.p. at a dose level of 0.02 mg. per 100 gm. of body weight for four successive hourly intervals 10% urethane given i.p. every other day at a dose level of 750 mg. per kg. for a total of eight injections.

ascorbic acid content (mg. per 100 gm. of fresh adrenal tissue) are presented in Table 1 for each of the five groups of rats used in this study. It is apparent from an inspection of Table 1 that there is no obvious functional alliance between the adrenal ascorbic acid with either the lymphocytes or the polymorphs in any single group of rats. For instance, three hours after the adrenaline treatment (Group *b*), there was a statistically significant depletion of adrenal ascorbic acid in these rats when compared with the controls; but there also was a statistically significant depression of the lymphocytes and increase in the circulating number of neutrophils. However, when these different

TABLE 2. THE CORRELATION COEFFICIENT AND THE SIGNIFICANCE OF THE COEFFICIENT OF THE ADRENAL ASCORBIC ACID WITH THE ABSOLUTE NUMBER OF CIRCULATING LYMPHOCYTES OR POLYMORPHO-LEUCOCYTES IN THE NORMAL INTACT, THE ADRENALINE TREATED, AND UNRETHANIZED RAT

Animal used	Adrenal ascorbic acid with the absolute number of Lymphocytes		Adrenal ascorbic acid with the absolute number of Polymorphs	
	"r"*	"p"***	"r"	"p"
NORMAL:				
a. Controls (16)	0.0109	>0.05	0.5754	<0.05
b. 3 hours after*** adrenaline (14)	0.0928	>0.05	0.1488	>0.05
c. 20 hours after adrenaline (14)	0.2690	>0.05	0.6121	<0.05
URETHANIZED:				
d. Controls (8)	0.4315	>0.05	0.8899	<0.01
e. 3 hours after adrenaline (9)	0.3543	>0.05	0.0440	>0.05

* "r" is the correlation coefficient

** "P" is the value of the correlation coefficient occurring by chance alone.

*** Adrenaline given sub-Q at a dose level of 0.02 mg. per 100 gm. of body weight for four successive hourly intervals.

10% urethane given I.P. every other day at a dose level of 750 mg. per kg. of body weight for a total of eight injections.

Number in () is the number of rats in the experiment.

categories of data are followed and statistically compared with each other in Groups *a*, *b*, and *c* it is apparent that the pattern of change of the adrenal ascorbic acid content is more closely followed by the pattern of change of the polymorphs than the lymphocytes. These data suggest that with adrenaline treatment the increase and return to normal number of circulating polymorphs was related to the depletion of adrenal ascorbic acid and return to normal amount. The correlation coefficient "r" and the statistical significance "P" of the coefficient was determined in each of the groups of rats for the correlation of the adrenal ascorbic acid with the lymphocytes and with the polymorpho-leucocytes (Table 2). Linear regression curves were calculated from the individual data of each group of rats and plotted according to the regression formula $Y = a + bX$ (Snedecor, 1946). These curves indicate the trend of the correlation between the adrenal ascorbic with the polymorphs or with the lymphocytes (Figures 1, 2, and 3).

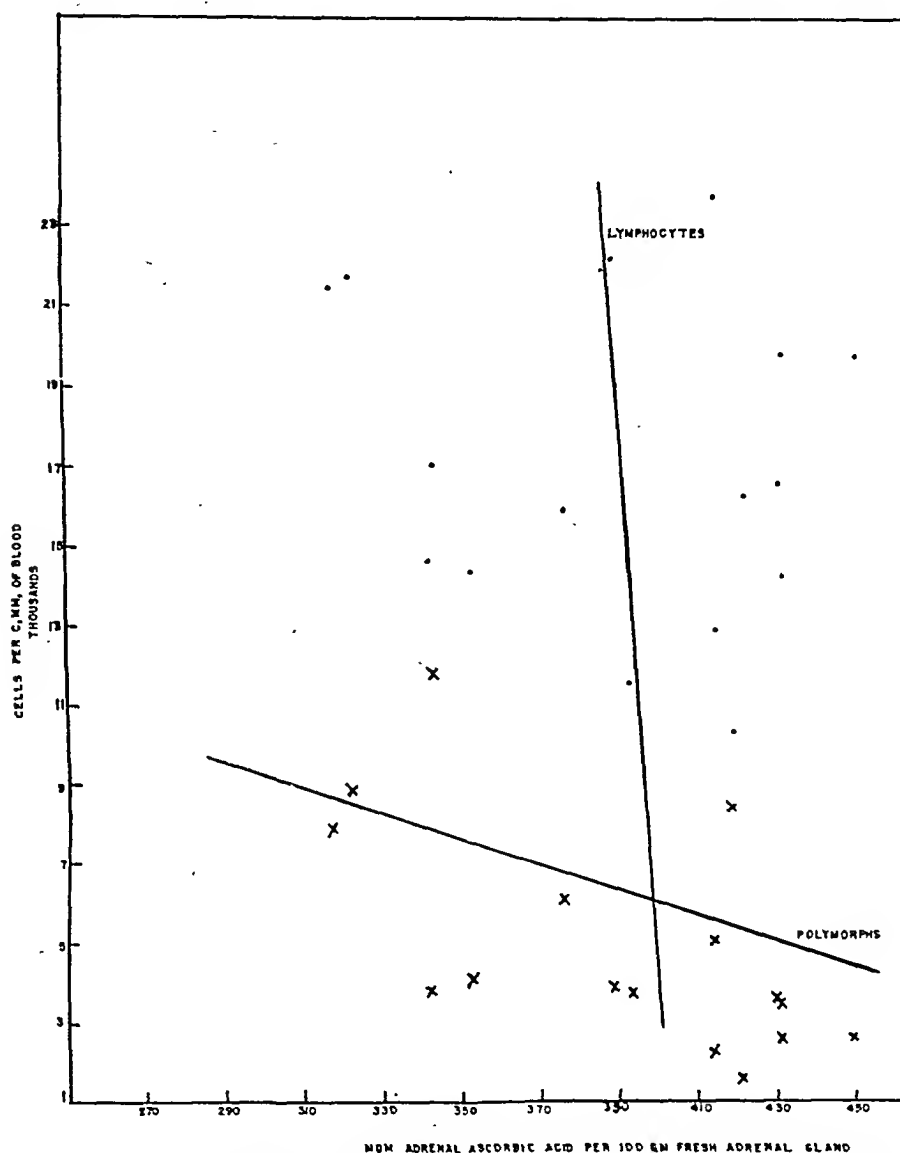


FIG. 1. The correlation distribution of the ascorbic acid content of fresh adrenal tissue with the absolute number of circulating lymphocytes and polymorpho-leucocytes per c.mm. of blood in normal intact rats. The solid lines are calculated linear regressions of the lymphocytes and the polymorphs on the adrenal ascorbic acid.

It is evident from an inspection of Figure 1 that the absolute number of circulating lymphocytes is randomly distributed and not correlated with the adrenal ascorbic acid content at the moment of autopsy. The absolute number of circulating polymorpho-leucocytes is negatively correlated with the adrenal ascorbic acid at the moment of autopsy and is statistically significant in the normal intact rat (Table 2).

had apparently not been affected. It is noteworthy that in this group of urethanized rats that the correlation coefficient of the adrenal ascorbic acid with the polymorpho-leucocytes is statistically significant (Table 2) as was with the controls. Reference to Figure 4 shows that the linear regression curves for the adrenal ascorbic with the polymorphs and with lymphocytes are negative. It would also appear that the correlation of the adrenal ascorbic with the lymphocytes

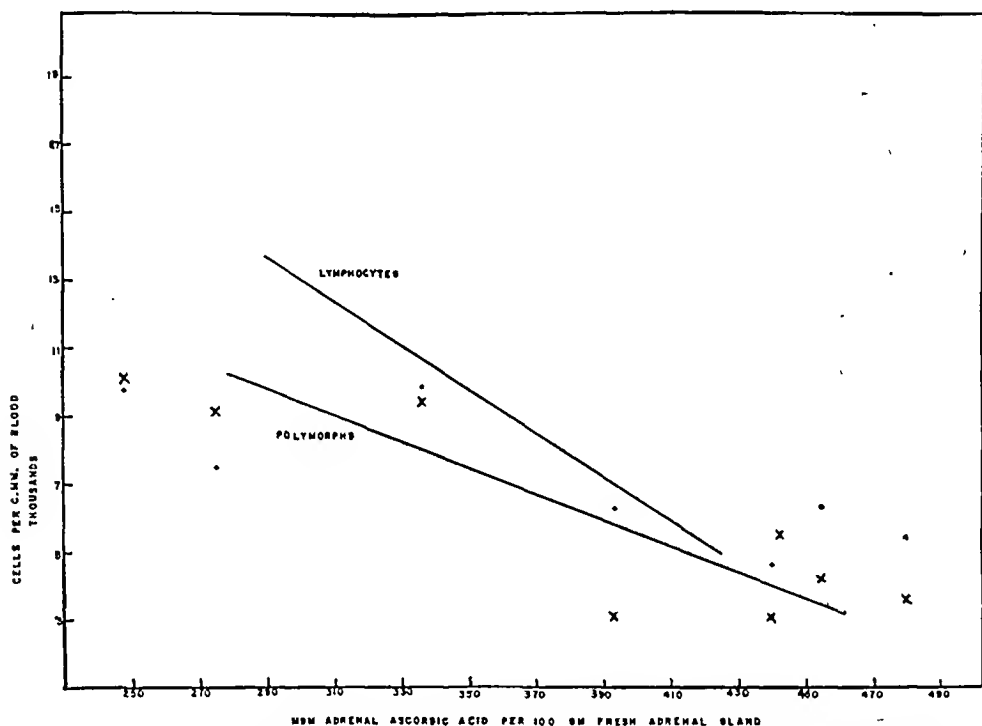


FIG. 4. The correlation distribution of the ascorbic acid content of fresh adrenal tissue with the absolute number of circulating lymphocytes and polymorpho-leucocytes per c.mm. of blood in the urethanized rat. The solid lines are calculated linear regressions of the lymphocytes and the polymorphs on the adrenal ascorbic acid.

might be significant; but statistical treatment of the data shows that this is not true (Table 2).

It had been shown by Dury and Robin (1948) that adrenaline injections in urethanized rats resulted in a neutrophilia which was still present twenty hours after the last adrenaline injection. These investigators suggested that urethane's effect was not limited to the circulating lymphocytes alone but to the polymorphs as well probably through the adrenal mechanism. In this study the rats comprising Group *c* were urethanized and then treated with adrenaline as described in the section on Methods. A comparison of the data of Group *c* with that of Groups *d* and *b* (Table 1) shows that the adrenaline had been effective in depressing the lymphocytes and increasing the circulating number of polymorpho-leucocytes. However, this apparently

did not result in a depletion of the adrenal ascorbic acid in the urethanized group of rats as had occurred in the normal rats after the adrenaline injections. The change in the correlation distribution of both the lymphocytes and the polymorphs three hours after the adrenaline injections is shown in Figure 5. The correlation coefficient of the polymorpho-leucocytes with the adrenal ascorbic acid was not statistically significant (Table 2). Since there was a statistically significant neg-

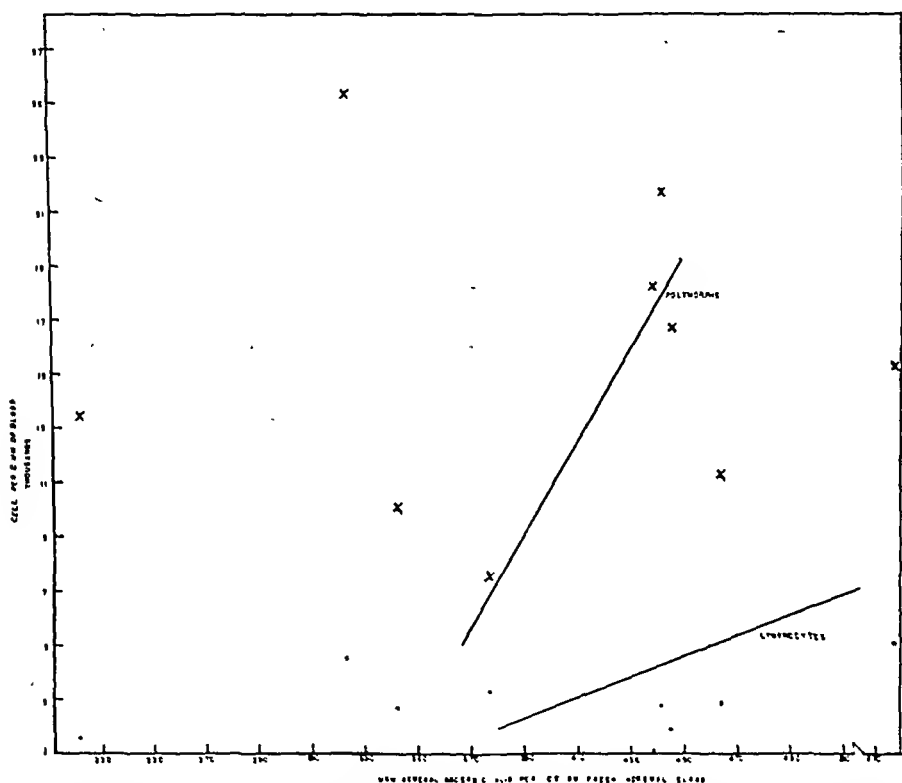


FIG. 5. The correlation distribution of the ascorbic acid content of fresh adrenal tissue with the absolute number of circulating lymphocytes and polymorpho-leucocytes per c.mm. of blood in the urethanized rat three hours after a course of adrenaline injections. The solid lines are calculated linear regressions of the lymphocytes and the polymorphs on the adrenal ascorbic acid.

ative correlation of the polymorphs with the adrenal ascorbic acid in the urethanized series of rats (Figure 4, and Table 2), the evidence from the urethanized-adrenaline treated rats is suggestive that the adrenaline injections had been effective via the adrenal-cortical mechanism. The fact that the adrenal ascorbic acid content remained at apparently normal level three hours after the adrenaline injections of these urethanized rats appears to support previous evidence of the paradoxical effect of urethane on the constituent cell types of the

white blood cell picture, and is believed to be further evidence that urethane is somehow related to its effect on the neutrophiles through the adrenal cortex.

DISCUSSION

Though the adrenal ascorbic acid has been proposed as a useful tool in the measurement of adrenal-cortical activity (Sayers, Sayers and Woodbury, 1948), it is not known exactly what role it actually has in the endocrine function of the adrenal cortex (Long, 1947). The data presented here does not assign any role to the adrenal ascorbic acid. However, it is evident from the data that there exists in the rat a functional relationship between the ascorbic acid present in the adrenal gland and the number of circulating polymorphonuclear leucocytes (in the rat this is essentially neutrophiles). This does not contradict the evidence presented by other investigators that the adrenal ascorbic acid may be used as an indicator of adrenal-cortical activity associated with lymphocyte dissolution. However, it is recognized that there is apparently no chemical relationship between the adrenals' vitamin C and the adrenal-cortical hormones.

Attention has already been called to the fact that a neutrophilia has always been reported as coexistent with a lymphopenia in the first few hours after adrenal-cortical stimulation with ACTH or adrenaline injections. The interesting aspect of the data presented here is that in the normal rat a statistically significant negative correlation has been shown to exist between the adrenal ascorbic acid and the absolute number of circulating polymorphs whereas this has not been found for the lymphocytes. This correlation of the adrenal ascorbic acid with the polymorpho-leucocytes has been supported by experimentally inducing changes in both the lymphocytes and the polymorphs simultaneously or separately with adrenaline and/or urethane and following the correlation of these white blood cells with the adrenal ascorbic acid. At no point in the experimental procedures followed was a statistically significant correlation found for the adrenal ascorbic acid with the lymphocytes; but the correlation of the polymorphs with this vitamin followed the expected pattern of relationship in adrenal-cortical stimulated animals when this had been shown to exist in the normal intact rat (Table 2). If the fact is accepted that adrenaline induces increased adrenal-cortical activity which was measured by the ascorbic acid content of the gland, then it is evident that at the point of maximum activity the physiological effect will not be correlated with the glands' ascorbic acid content. As the adrenals recoup toward a state of physiological normality there would occur a re-establishment of a normally existing regulation as indicated in this case by a correlation of the polymorphs with the adrenal ascorbic acid. An inspection of Figures 1, 2, and 3 illustrates the shifting of the polymorpho-leucocytes correlation population with relation to the adrenal ascorbic acid in the normal rat to one of complete non-correlation

three hours after adrenaline injections, and then a return to approximately the normal correlation twenty hours after adrenaline stimulation of the adrenal mechanism.

The urethanized series of rats add circumstantial and inferential evidence to the demonstrated correlation of the polymorphs with the adrenal ascorbic acid in normal rats. This is certainly illustrated by the data in Table 1 showing that the adrenal ascorbic acid is not necessarily lowered when there is a lymphopenia. Adrenaline injections can result in urethanized rats in still further depressing the number of circulating lymphocytes and also induce a neutrophilia (Table 1). These changes would indicate that the adrenal-cortex has been involved. However, in this series of rats the adrenal ascorbic acid had not been altered from the normal content. This phenomenon cannot be explained, but it is being investigated since it is suggestive of an alteration in urethanized rat of the "affinity" or "mobilization" of ascorbic acid in the adrenal gland when stimulated to increased activity.

The evidence presented in this study of the ascorbic acid correlation with the polymorphonuclear leucocytes does not mean this *a priori* has been a demonstration of the regulation of this white blood cell type by a vitamin found in high concentrations in the adrenal gland. The fact must be borne in mind that the adrenal ascorbic acid has been adequately demonstrated to be an indicator of adrenal-cortical activity. It is most probable therefore that the material in this study indicates a relationship of one of the adrenal cortex secretions to the regulation of the circulating neutrophiles. However, since the role and relationship of the vitamin C content of the adrenals to its endocrine function is not known, there is a possibility that these data are indicative of a vitamin-humoral regulation of a physiological process. This aspect of the problem is under study at present in both the rat and the guinea pig.

SUMMARY

Data have been presented which show that there is a statistically significant negative correlation of the adrenal ascorbic acid content with the number of circulating neutrophiles in the normal rat. There was no evidence of a correlation of the lymphocytes with the adrenal ascorbic acid other than the fact that following adrenal-cortical stimulation the induced lymphopenia was associated with a depletion of adrenal ascorbic acid.

The leucocytic picture was actively influenced by adrenaline injections which have been shown to be mediated via the adrenal-cortical mechanism. The adrenal ascorbic acid was found to be depleted three hours after the adrenaline injections but there was no correlation of either the lymphocytes or the polymorpho-leucocytes with the adrenal ascorbic acid. However, twenty hours after the adrenaline injections the correlation of the circulating polymorphs with the ascorbic acid had been reestablished.

Urethanization induced a lymphopenia, but did not alter the circulating number of neutrophils nor affect the adrenal ascorbic acid. The correlation of the latter were found to be statistically significant, and the linear regression curve of the polymorphs on the adrenal ascorbic was similar to that of the normal intact rat. Urethane, however, did change the apparent effect of adrenaline on the adrenal ascorbic acid. The level of concentration of the vitamin C in the adrenal three hours after adrenaline was the same as in the normal gland although the leucocytic response was similar to that of the normal intact rat.

The data suggest that the adrenal cortex is responsible to an as yet unknown extent in the regulation of the number of circulating neutrophils in the normal rat. The place of the adrenal ascorbic acid in this regulation is discussed. It has been suggested that urethane might be a useful tool in determining the role of the adrenals' vitamin C in the white blood cell picture of the normal rat.

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NOTES AND COMMENTS

PREGNANCY TEST WITH MALE *BATRACHIA*

THE PREGNANCY test using male *Batrachia* as reacting animals was first performed by Galli-Mainini, who in March 1947 published his first series of 179 diagnostic and control tests. This reaction, when positive, is shown by the presence of spermatozoa in a drop of the batrachian's urine, within 3 hours after the subcutaneous injection of untreated woman's urine. This new test seemed to have the advantage of specificity, speed of reaction, simplicity and economy and it has aroused the interest of many investigators, with the result that today, a year since the original publication, 27 papers on the subject have been published, with a total of 3,156 diagnostic tests and 2,108 control tests performed. In addition, a great deal of experimental work has been done on the influence of several factors on the reaction and on its mechanism. Detailed data on results will be published elsewhere and it can be summarized as follows:

Correct results: On a total of 3,156 diagnostic tests, correct positives have varied from 98.16% to 100%, according to different authors.

Specificity: The specificity is shown by 100% correct negative results on 2,108 control reactions performed by injecting non-pregnant woman's urine. Injection or implantation of more than 45 substances, purified hormones or tissues, showed that only some gonadotrophins elicit the reaction, among which chorionic gonadotrophin seems to be one of the most effective.

Comparative results with the Friedman test: On 502 simultaneously performed reactions, 42 were not coincident, of which 38 correct responses were given by the toad and 4 by the rabbit.

Speed of the reaction: Practically 100% of responses are obtained within 3 hours and 80% in two hours. Some are positive in 30 minutes.

Precocity: Correct positive reactions have been obtained in 80 tests with urine from pregnant women with less than 15 days of amenorrhea, among whom were 23 with amenorrhea from 4 to 9 days and 21 with amenorrhea of 10 days.

Toxicity: Galli Mainini's data, on a total of 3,054 diagnostic or control tests performed show 0.76% mortality as result of the urine injection.

As some investigators are under the impression that this test is the peculiar reaction of only a special type of toad, *Bufo arenarum* Hensel, or frog, *Rana pipiens*, it seems advisable to communicate that several male *Batrachia* have already been used with good results. They are: *Bufo arenarum* Hensel, *Bufo paracnemis*, *Bufo crucifer*, *Bufo d'Orbigny*, *Bufo marinus*, *Calyp- tocephalus gayi*, *Leptodactillus ocellatus*, *Odontophrynus*, and only lately, *Rana pipiens*.

We think that this reaction could be a common phenomenon of a great number of other male batrachians, providing they have a continuous spermatogenesis. Seasonal unresponsiveness can be found in some *Batrachia* with

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cyclic spermatogenesis. In these animals spermatozoa are produced only during a certain period, the testicle being at rest between the active seasons. For this reason, care should be taken in selecting an animal with continuous spermatogenesis. If so, it seems possible that many other male *Batrachia*, when injected with pregnancy urine, would respond in the same manner as the previously mentioned, thus providing a reliable test animal throughout the year. We are under the impression that toads are sturdier animals than frogs because larger quantities of urine can be injected, the mortality is less and the reaction can be repeated several times in them.

As a scarcity of bibliographic references on the subject has characterized some of the latest papers, it seems advisable to provide the available bibliography up to the present for the benefit of investigators interested in this new reaction.

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ASSOCIATION NOTICE

ANNOUNCEMENT OF THE 1949 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-First Annual Meeting of The Association for the Study of Internal Secretions will be held in the Chalfonte-Haddon Hall, Friday and Saturday, June 3, and 4, 1949, in Atlantic City, New Jersey.

We are informed by the hotel management that reservations will be difficult to secure on short notice; therefore, members are urged to make reservations at once with Chalfonte-Haddon Hall, giving time of arrival and length of stay in Atlantic City.

The scientific sessions will be held in the Viking Room, as formerly, and registration will be on the same floor. The annual dinner will be held in the Rutland Room, Friday, June 3rd. at 7 P.M., preceded by cocktails in the same room.

Those wishing to present papers, which will be limited to ten minutes, should send title and four copies of an abstract of not more than 200 words, to Doctor J. S. L. Browne, Royal Victoria Hospital, Montreal 2, Canada, not later than March 1, 1949. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program.

Nominations for the Squibb and Ciba Awards and the Ayerst, McKenna and Harrison Fellowship should be made on special application forms, which may be obtained from the Secretary-Treasurer, Doctor Henry H. Turner, 1200 North Walker, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1949.

POSTGRADUATE COURSE IN ENDOCRINOLOGY

A postgraduate course in Endocrinology, sponsored by the Association for the Study of Internal Secretions, will be held at the Skirvin Hotel in Oklahoma City, February 21-26, 1949.

The faculty will consist of outstanding clinical and research endocrinologists of the United States and Canada. The program will consist of clinics and demonstrations and will be a practical one of equal interest to those in general medicine and the specialists.

The fee will be \$100 for the entire course and applications will be accepted in the order received. Applications should be directed to Henry H. Turner, M. D., Secretary-Treasurer, 1200 North Walker, Oklahoma City, Oklahoma.

NOTES ON THE THIRTIETH ANNUAL MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirtieth Annual Meeting was held in the Palmer House, Chicago, Illinois, June 18 and 19, 1948.

Forty-seven papers were presented and forty-four papers were read by title. Total registration was 458, equally divided between members and non-members. Two hundred, twenty-two were present at the dinner at which the presidential address was given by Doctor C. N. H. Long.

Actions of general interest taken by the Council are as follows:

(1) It was the decision of the Council to hold a Postgraduate Assembly in Oklahoma City, February 21-26, 1949, similar to the one which was so well received in Los Angeles in 1948.

(2) The Council approved the indexing and publication of *ENDOCRINOLOGY*, Volumes 1 to 40, inclusive. These will be published by Mr. Charles C Thomas, in two editions, one including the indices of Volumes 1 to 25, and the other, Volumes 26 to 40.

(3) It was the Council's decision to publish the transactions of the American Goitre Association.

(4) The Council accepted with much regret the resignation of Doctor Earl T. Engle, Chairman of the Publications Committee, and appointed Doctor Warren O. Nelson to this office. Appreciation was expressed to Doctor Engle for his work as chairman of this committee.

(5) The Committee on Registry of Endocrine Pathology was re-appointed to negotiate with the Scientific Director of the American Registry of Pathology regarding the formation of an Endocrine Registry at the Army Institute of Pathology. A sum not to exceed \$750.00 was appropriated for necessary expense for one year.

(6) It was voted to appropriate \$250.00 for 1949 to the National Society for Medical Research.

(7) Due to increasing cost of labor, paper and all printing materials, and the enlargement of the Journals, the dues were increased to \$11.00 per year which includes subscription to either of the Journals, with a combination offer of \$16.00 for both Journals.

(8) The Council voted that the thirty-first Annual Meeting be held June 3 and 4, 1949, in Atlantic City, New Jersey.

(9) A list of the 1948-49 Officers, Council and Committees follows:

Officers

President	John S. L. Browne
President-Elect	Edward A. Doisy
Vice President	James H. Means
Secretary-Treasurer	Henry H. Turner

Council

Frank A. Hartman	Roy G. Hoskins
Carl R. Moore	E. Perry McCullagh
Gregory Pincus	Fuller Albright
C. N. H. Long	Mayo Soley
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*Committee on Postgraduate Course
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Lawson Wilkins

Committee on Nomenclature

Elmer Sevringhaus, Chairman
Hans Selye
Thomas McGavack

Consultant Committee to Food and Drug Administration

A. Kenyon, Chairman
George Sayers
J. P. Pratt

ASSOCIATION AWARDS FOR 1949

THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb & Sons Award of \$1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner; 1941—Dr. Philip E. Smith; 1942—Dr. Fred C. Koch; 1943—no award was given; 1944—Dr. E. A. Doisy; 1945—Dr. E. C. Kendall; 1946—Dr. Carl G. Hartman; 1947—Drs. Carl F. and Gerty T. Cori; 1948—Dr. Fuller Albright. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russel; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl G. Heller. The work cited may be either in the field of preclinical or clinical endocrinology. The Award is for \$1,200.00. If within 24 months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, the Award will be increased to \$1,800.00.

THE AYERST, McKENNA & HARRISON FELLOWSHIP

The Ayerst, McKenna & Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin, and in 1948 to Dr. Ernest M. Brown, Jr. This Fellowship is designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. The Fellowship may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend for the Fellowship will vary in accordance with the qualifications of the appointee, but will not exceed \$2,500.00. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. The nominee must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals; submit a program of proposed study; indicate one or more institutions where the proposed program shall be carried out; submit statements of approval from the investigators with whom he proposes to conduct his research.

Each member has the privilege of making one nomination for each award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to endocrinology and by a bibliography of the nominee's most important publications, and reprints, if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 15, 1949.

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A CYTOLOGICAL EXAMINATION OF UTERINE GROWTH DURING PREGNANCY

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THE PRESENT paper aims to extend the Karyometric statistical method for the analysis of interphasic nuclear growth to a study of uterine tissues. The endocrine viewpoint was kept in mind in this paper, which deals with uterine growth in pregnancy, as also in another paper (in press) which concerns a similar cytological study of uterine growth during the estrous cycle and artificially induced estrus.

The growth response, both in size and number, of all cellular elements of the uterus during pregnancy has long been known. Gander (1930), Stieve (1929, 1932), Froböse (1934, 1935), Fabris (1935), Crandall (1938), Krichesky (1942) and others conducted their researches from the standpoint of the cell as a whole, although they do mention increase in nuclear size. It is this latter phenomenon of which the Karyometric method takes advantage.

It is believed that such a study applied to the pregnant uterus, reflecting cycles of hyperplasia and hypertrophy of the tissues, would have endocrine significance.

METHOD

Albino rats of the Instituto Butantan were used in these experiments. They were killed on days 7, 13, 14, 20 or 21 of pregnancy; the uteri were fixed in Dubosq—Brasil fluid and imbedded in paraffin. The 10μ sections were stained in Haidenhain's hematoxylin and eosin.

From each representative zone 200–300 nuclei were sketched with the aid of a camera lucida at a magnification of 1890. Care was taken to select such areas in which the section passed through the long axes of the elliptical nuclei, thus avoiding errors of measurement due to inclination of the nuclear axes. In the tables the nuclei taken from the dilated parts of the uteri are

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indicated by the letter *a* after the protocol number, the letter *b* referring to the intermediary portions. Myometrial cells of the dilated portions were measured after removal of the fetus (indicated by *c* in the tables).

Inasmuch as the Karyometric statistical method here used has not appeared in the English language, it is here described in some detail (cf. Schreiber, 1947, a, b; 1948; Schreiber and Salvatore, 1947).

Determination of Nuclear Volume. The nuclear volume of an ellipsoid was calculated after the formula:

$$V = \frac{a^2 \times b}{1.91}$$

in which *a* represents the transverse, *b* the longitudinal diameter.

To illustrate, take item 39b of Table III. It was found that 3 nuclei measured 27×5 mm., and, by the above formula, volume was found to be 353.4 mm.³; of 5 other nuclei, one measured 33×5 mm., volume 431.9 mm.³;

TABLE A. (Prot. 39b of Table III):

N° of nuclei		Long axes	Short axes	mm. ³ (nuclear volume)	Classes	Interval of 50 mm. ³
3		27 mm.	× 5 mm.	353.4	between 350-399	mean 375
5	1	33	× 5	431.9	between 400-449	mean 425
	4	32	× 5	418.8	between 400-449	mean 425
6		25	× 6	471	between 450-499	mean 475
1		29	× 6	546	between 500-549	mean 525
24	9	30	× 6	565	between 550-599	mean 575
	15	31	× 6	584	between 550-599	mean 575
34	10	32	× 6	603	between 600-649	mean 625
	9	33	× 6	621	between 600-649	mean 625
	15	24	× 7	615	between 600-649	mean 625
20	12	27	× 7	692	between 650-699	mean 675
	8	26	× 7	667	between 650-699	mean 675
6		28	7	718	etc.	mean 725
8	6	23	× 8	770	mean 775
	2	22	× 8	750	mean 775
20	13	32	× 7	820	mean 825
	7	25	× 8	837	mean 825
38	22	26	× 8	871	mean 875
	10	34	× 7	872	mean 875
	6	35	× 7	897	mean 875
8	3	36	× 8	819	mean 925
	5	37	× 7	949	mean 925
	5	37	× 7	949	mean 925
6	4	38	× 7	974	mean 975
	2	29	× 8	971	mean 975
4	3	30	× 8	1005	mean 1025
	1	31	× 8	1038	mean 1025

Fig. 1A

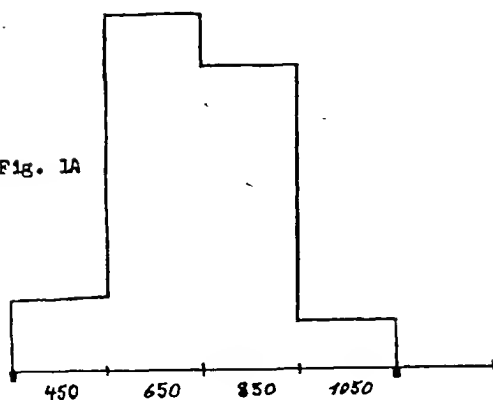


Fig. 1B

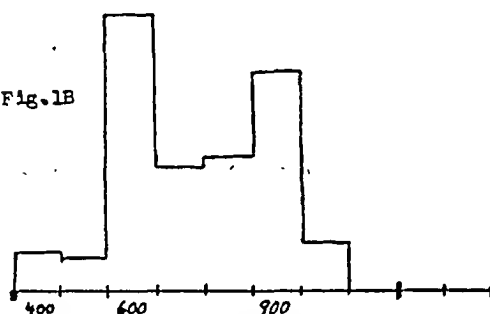


Fig. 1C

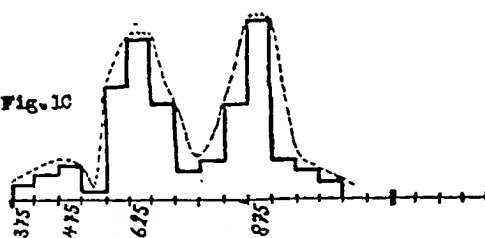
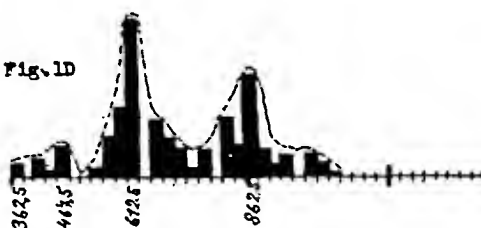


Fig. 1D



FIGS. 1A-D. Histograms illustrating the result of graphing the data for case 39b (Table III) according to different class intervals: 1A, 200 mm.³; 1B, 100 mm.³; 1C, 50 mm.³ and 1D, 25 mm.³. The histograms are drawn on different scales.

4 nuclei, 32×5 mm., had a calculated volume of 418.8 mm.³ and so on, as shown in Table A. Absolute values can be calculated if one wishes; one mm. corresponding to 0.53 micron; but this has not been considered necessary, as the study is one of relative volume variations.

Statistical Elaboration of Results. In this Arkin and Colton's Outline of Statistical Methods, 4th edition, Barnes and Noble, Inc., New York, 1942, served as a guide.

The values found (nuclear volumes) were grouped in frequency classes in ascending series of sizes of nuclei, as may be seen in Table III, for example. The class interval here chosen, for reasons to be discussed below, is 50 mm.³ Thus all nuclei having volumes between 400 and 449 were collected under one class represented by the average value 425; all between 450 and 499 were collected under the average value 475 and so on (Table A).

TABLE B. (Prot. 39b of Table III):

Classes of 50 mm. ³ (or 0.50):													
375	425	475	525	575	625	675	725	775	825	875	925	975	1,025
3	5	6	1	24	34	20	6	8	20	38	6	4	etc.
													Frequency (Fig. 1C)
Classes of 100 mm. ³ :													
400	500	600	700	800	900	1,000							
8	7	58	26	28	46	10							
													etc.
													Frequency (Fig. 1B)
Classes of 200 mm. ³ :													
450	650	850	1,050										
15	34	74	10										
													etc.
													Frequency (Fig. 1A)

Class intervals of 25 mm.³, 100 mm.³ and 200 mm.³ might have been used as shown for protocol 39b in Table B. The superiority of the 50 mm.³ interval for Table III will be apparent from a perusal of the frequency histograms shown in Figure 1A to 1D.

Figure 1A demonstrates the fact that with a class interval of 200 mm.³ only one modal class (at 650) emerges. With an interval of 100 mm.³ three modal classes are noted, namely at 400, 600 and 900 mm.³ (Fig. 1B). Greater neatness of the three modal classes (at 475, 625 and 875) is seen when the class interval of 50 mm.³ is utilized, as in Figure 1C, which represents case 39b of Table III (cf. Table B). The 50 mm.³ class interval has, therefore, been employed for the nuclear volumes of muscle cells (Table III). For glandular and endometrial cells, whose volumes are greater, by the same method of analysis, the 100 mm.³ class interval was chosen.

It is apparent that the probabilities of mistake is reduced in proportion to the reduction in the class interval (Arkin and Colton). Thus the 25 mm.³ interval (Figure 1D) gives even a better curve than the 50 mm.³ interval but not enough better to warrant the space necessary for presenting double the number of classes. Fifty mm.³ would, therefore, seem both accurate and practicable.

Further analysis of our data is presented in Figure 2, which reports average histograms made up of the arithmetical mean of the nuclear volumes of day 7, 13, 14 and 20, 21, respectively. The calculations were made from Table III as follows:

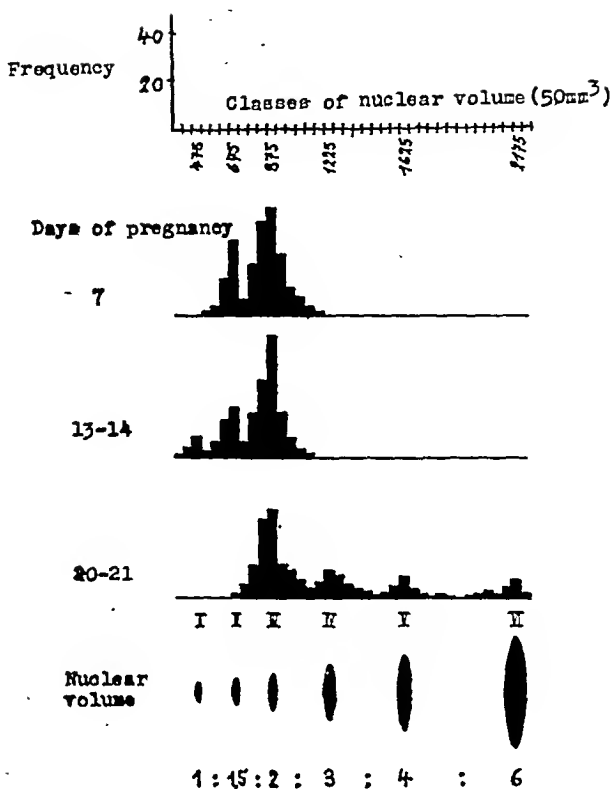


FIG. 2. Average histograms of nuclear volumes of muscle cells at three stages of pregnancy (see Table III); on the ordinates, nuclear volume frequencies; on the abscissas, class intervals. Below, schematic diagrams of nuclear sizes.

The nuclei of each class pertaining to the same time of gestation were added and the sum divided by the number of cases involved. Thus in the 525 mm.³ class, there is one nucleus of case 36a and two of 36b, a total of 3 nuclei in two cases, an average of 1.5. In class 575 the average calculates to 4 (12 nuclei in three cases).

Because of lack of space no table has been presented of these calculations—the histograms of Figure 2 must suffice to show the trends. Under the histograms, the nuclear volumes are represented schematically.

Determination of Modal Value. In these calculations of modal values, Arkin and Colton's formula (p. 23) has been followed:

$$M = L + \left(\frac{F}{F + f} \times i \right).$$

To illustrate with case 39b of Table III which reveals 3 maxima of frequency (i.e. 3 modal classes): 475, 635 and 875 mm.³ (cf. Fig. 1C): In this case the first modal class is that of 475 mm.³ (Figure 3).

L = the lower limit of the modal class, i.e. that between 425 and 475, or 450 mm.³

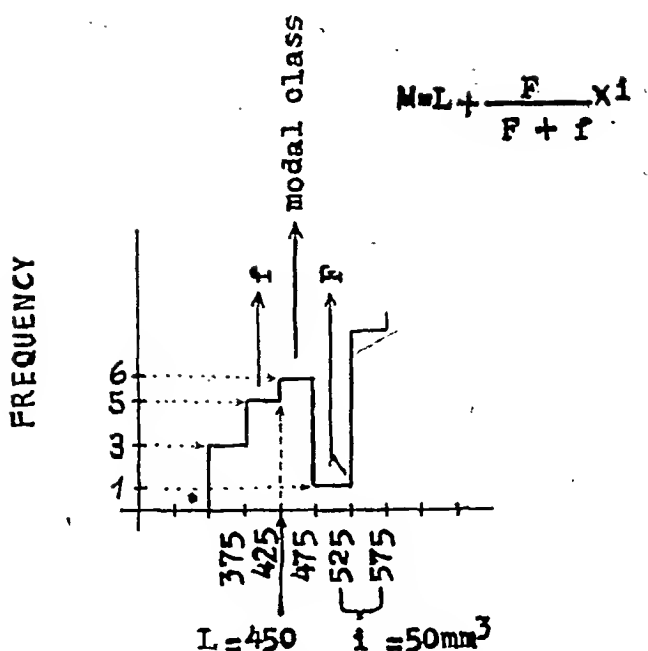


FIG. 3. To illustrate calculation of the modal value (M). See test.

F = the frequency of the class above the modal class; in our example it is represented by one nucleus ($F = 1$).

f = the frequency of the class below the modal class, namely 5 ($f = 5$).

i = the class interval, which in the case of the myometrium (Table III) is 50 mm.³

Substituting:

$$M = 450 + \left(\frac{1}{1 + 5} \times 50 \right) = 458$$

458 therefore, represents the modal value of the first class of maximal frequency for the individual case selected, namely 39b of Table III.

The second modal value is calculated thus:

$$M = 600 + \left(\frac{20}{20 + 24} \times 50 \right) = 622$$

For case 39b we find modal values 458, 622 and 864.

$864 \div 458 = 1.88$ or approximately 2

$864 \div 622 = 1.38$ or approximately 1.5

Pursuing the calculations further we arrive at figures closely approximating the theoretical numbers of the progression: 1: 1.5: 2: 3: 4: 6 etc.

For the sake of brevity only Tables I, II and III are presented. These show the frequencies of the calculated nuclear volumes and the calculated modal values, the fundamental values in this research.

The mitotic indices and the degree of infiltration of leucocytes are also presented in the tables.

The reality of the modal values, in the purely statistical sense, is proven not only by the constancy of the analyzed cases but also by comparison of

TABLE 1. UTERINE LINING EPITHELIUM

N. of proto-col	Days of preg-nancy	Modal value				Nuclear volume																		N. of nuclei	Mitotic index	Leukocytic infiltration	
		I	II	III	IV	350	450	550	650	750	850	950	1050	1150	1250	1350	1450	1550	1650	1750	1850	1950	2050				
15 a	7		1061	1432						4	10	20	10	20	42	60	20	5							197	—	—
15 b	7			1437							5	10	24	38	55	23	7								172	—	—
36 a	7		1061	1301.						2	2	5	10	8	25	45	40	20.			1				167	—	—
36 b	7			1430							4	8	10	30	35	50	15	8							166	—	—
38 a	13	657	1050	1431	1033	2	3	9	23	12	10	10	19	10	13	31	40	14	10	2	2	6	1		217	4.3	+
38 b	13	654	1037	1430	1900					8	10	25	6	14	28	56	12	7	5	1	9				182	3.1	+
39 a	14	665	1047	1438			2	5	15	6	13	10	18	9	21	30	42	19	8	3					215	3.3	+
39 b	14	636	1036	1371			4	7	18	13	12	15	7	16	45	40	12	4	1						233	2.4	+
47 a	20			1454			5	21	38	12	5	1	5	14	13	27	60	32	9	1					162	—	+
17 b	20		1440								2	8	24	42	76	28	5	2							187	—	+
2 a	20			1444	1037							5	12	30	74	24	13	5	10	3					200	—	+
2 b	20			1443	1906							2	10	26	62	20	13	8	1	5	2				149	1.7	+
22 a	21		1447	1350								1								5	1	3	1		176	3.9	+
22 b	21			1423	1900							2	8	32	40	10	3	3			4	8	0		116	5.7	+

TABLE 2. GLANDS

N. of pro- col	Days of preg- nancy	Modal value				Nuclear volume																		N. of cel- el	Mito- tic in- dex	Leuko- cyto- infiltra- tion			
		I	II	III	IV	250	350	450	550	650	750	805	950	1050	1150	1250	1350	1450	1550	1650	1750	1850	1950				2050	2150	2250
15 a	7			1363								5	12	35	68	60	29	9									218	—	—
15 b	7		1052	1422							8	12	9	28	47	60	14	8									170	—	—
30 a	7		1100	1415					2			10	6	27	33	64	27	5									174	—	—
30 b	7		1127	1452							1	8	16	30	6	27	50	30	10								168	—	—
38 a	13	680		1418							5	25	20	8	5	30	40	28	4								175	—	—
38 b	13	680	900	1425							10	9	7	15	45	47	15	2									180	+	+
39 a	14	688	1026	1424							25	23	43	40	7	25	36	8									190	+	+
39 b	11	900	988	1362							3	25	24	12	21	36	35	15	4								216	—	—
17 a	20			1430			1833				5	10	23	35	62	71	27	7		2	4						252	0.6	+
17 b	20			1360							3	6	16	40	69	61	16	10	5								230	—	—
2 a	20			1435			2066					1	23	36	41	100	23	13	8								238	0.4	+
2 b	20			1431			1974					2	32	30	80	94	30	14	5								315	—	+
22 a	21			1425			1014						10	32	58	89	20	8	5	1	6	10					231	4.7	+
22 b	21			1428			1087						8	30	45	69	18	5		—							181	5.2	+

TABLE 3.

N. of protocol	Days of preg- nancy	Modal value						Nuclear																
		I	II	III	IV	V	VI	375	425	475	525	575	625	675	725	775	825	875	925	975	1025	1075	1125	1175
15 a	7		655	870								2	8	15	1	18	35	50	25	16	13	3	2	
15 b	7		678	860									5	16	7	8	45	60	12	8	2			
36 a	7		666	829							1	4	25	50	12	28	46	40	27	7	5	3	2	
36 b	7		616	864							2	6	14	3	2	10	26	53	11	5	2			
38 a	13		662	866						3	4	6	19	28	6	10	27	51	13	6	2			
38 b	13		668	876								1	5	14	3	7	16	33	18	8	4	2		
39 a	14	460	626	868				1	8	16	2	13	16	15	10	29	39	55	22	12	4	3	2	
39 b	14	458	622	864				3	5	6	1	24	34	20	6	8	20	38	8	6	4			
17 a	20			829	1183									2	6	30	58	44	12	10	3	1	1	4
17 b	20			836	1183	1583									3	8	30	100	78	25	14	6	2	4
2 a	20			864	1216	1590	2133							5	8	18	42	45	17	15	10	5	6	10
2 b	20			883	1233	1618	2131									8	10	25	20	9	9	6	5	8
2 c	20			836	1222	1545	2162								5	6	20	16	12	3	9	2	3	15
22 a	21			870	1167	1576								2	4	7	18	40	12	11	11	13	15	26
22 b	21			833	1163	1580									2	20	44	40	32	15	16	8	22	27

all the maxima of frequencies pointed out in the tables; these maxima show a perfect correspondence among those values in the different stages studied.

It may further be pointed out to the reader that a modal class which may be called "Secondary" because it is made up of few nuclei in one phase of pregnancy, may become a "principal" modal class in another phase. This fact demonstrates the biological reality of the modal value, in correlation with the rhythmic growth of the studied material.

RESULTS

If we compare the 3 charts, the first thing that strikes us is that the variations of nuclear volumes in all tissues examined are similar, and that they take place simultaneously. Furthermore, the increase of the nuclear volume is not steady, but rhythmic, the modal values of which are proportional to a basic modal value. Besides this, the variations of nuclear volumes are exactly the same in non-dilated intermediary as well as in the dilated parts.

In the first part of these researches (1948) we recorded the variations of nuclear volumes during the estrous cycle and artificially induced estrus and showed that the lowest modal values found were 704 in the epithelium, 664 in the glands and 424 in the myometrium, which values we take to be our *basic values* (mode I).

We next observed on the 7th day of pregnancy a first mode of 1061 in the epithelium, 655-666 (a) and 678-616 (b) in the myometrium. All these figures corresponding roughly to value 1.5 of the above mentioned basic nuclear volume; the same was found during diestrus, metestrus and in castrated animals. This modal value, which is 1.5 times the basic modal value, we called mode II. The highest frequency of nuclear volumes was found in modal value 1432-1361 (a), 1437-1430 (b) in the epithelium and 870-829 (a), 829-864

MYOMETRIUM

volume																				N. of nuclei	Mitotic index	Leuko-cytic infiltration		
1225	1275	1325	1375	1425	1475	1525	1575	1625	1675	1725	1775	1825	1875	1925	1975	2025	2075	2125	2175	2225				
																					183	0.5	—	
																					163	—	—	
																					250	0.4	—	
																					134	—	—	
																					175	1.1	+	
																					111	—	+	
																					247	0.4	+	
																					183	—	+	
																					173	—	+	
2							1	10	2												307	—	++	
8	2	1	1				1	12	4	1		2	1		1	1	3	1	5	2	235	—	++	
12	5	2		1			2	18	59	11	20	6			3	5	3	6	12	10	2	296	0.9	++
19	16	9	7	6	2		1	12	9	4	1				1	7	2	12	21	4	220	—	++	
24	12	8	6	2	1		3	6	12	7	2										201	—	++	
8	3		1				3	6	12	7	2										259	—	++	
8	6	2	1		1	2	6	3		1				3		1								

(b) in the myometrium which correspond roughly to twice the basic value (mode III).

The glandular epithelium shows variations similar to those of the lining epithelium. There is neither infiltration of leucocytes, nor mitoses: only in the myometrium mitoses were some observed (mitotic index: 0.5 and 0.4%: a).

On the 13th and 14th day of pregnancy there appeared both in the endometrium and the myometrium, basic modal values (mode I) followed by mode II and also the mode which represents the highest frequency on nuclei, of twice the basic volume (mode III). A mode IV, at 3 times the basic value was observed in the glandular endometrium.

At this stage of pregnancy we found also histological changes similar to those of estrus, i.e., there is a typical infiltration of leucocytes and mitotic forms are present in the glands and superficial endometrium, as well as, in the myometrium (mitotic index 1.1 and 0.4%). On the 20th and 21st day of pregnancy the first mode observed represents values of 1454-1444 etc. in the endometrium, and 829-864 etc. in the myometrium which equal mode III (twice the basic volume), while there is a complete absence of lower values. On the other hand, besides mode IV that we found in both tissues, we found in the myometrium nuclear volumes of a modal value 4 times the basic value (mode V) (average 1592) and of a modal value 6 times the basic value (mode VI) (average 2188).

If we look at the histograms Figure 2 showing the myometrium, (the same holds for the endometrium) we find the variations of the nuclear volumes to be strictly rhythmic. We shall later refer to the ratios between the stages of the rhythmic growth.

On the 20th and 21st day of pregnancy, much infiltration of

leucocytes was seen in all uterine tissues: mitoses were found in the glands and superficial epithelium as well as in the myometrium.

DISCUSSION

The increase in size of the uterus during pregnancy results, as is now well known, chiefly from hypertrophy and hyperplasia depending on hormonal function and on the mechanical distention of the uterus, caused by the foetus it encloses.

We shall discuss separately the phenomena of hypertrophy and hyperplasia of the uterine elements and compare our data with those of the existing literature on the subject.

Hypertrophy: The increase in size of each individual uterine cell is generally admitted to be the main factor of uterine growth. Koeliker (1840), quoted by Crandall (1938), held there is an enlargement of muscle cells (hypertrophy) during pregnancy, as well as, hyperplasia. Hammond (1935), Reynolds (1937), Markee and Hinsey (1935) and Miyasaki (1938) observed an increasing thickness of the myometrium during gestation. Markee; Wills and Hinsey (1936) also observed an increase in size of the endometrium and of the connective tissue. Krichesky (1942), on the basis of his experiments with intra-ocular transplantation of uterine parts, held that individual hypertrophy of muscle fibres and interstitial swellings in the muscles, contribute to the enlargement of the myometrium. He further states that the superficial endometrium is also subject to hypertrophy during the first days of pregnancy. Crandall (1938) measured the volume of the muscle cells and found that the hypertrophy of these cells corresponds to $\frac{1}{2}$ or $\frac{1}{3}$ of the total increase of uterine volume.

From the cytological point of view, the variations of cellular volume during pregnancy were established almost exclusively with regard to muscle tissues. Stieve (1927, '29b) working with the human uterus, and Gander (1930) working on mice, measured not only the enlarged muscle cells but also their nuclei and proved that they also show variations in size.

Froböse (1932, '34, '35), examining uteri of rabbits and albino rats in various stages of pregnancy, observed that the cells as well as their nuclei grew steadily, reaching maximum size during delivery. He also observed (1932) that the muscle cells of the non-dilated uterine parts, did not attain to the same size but that their nuclei did, which means that the increase in nuclear volume was the same in all parts of the uterus.

Fabris (1935) examining uteri of cows, of pregnant women and cases of extrauterine pregnancy, obtained results similar to Froböse's both as to muscle cells and their nuclei. These two authors as well as Knaus (1929) also attribute these changes in nuclear size to hormonal agents.

As can be seen on the tables and on Figure 2 the results of our

research do not only prove the presence of nuclear hypertrophy (endometrium and myometrium) but they also show that this is due to hormonal agents, since the changes in the nuclear volume are exactly the same, and are simultaneous, in the dilated parts enclosing the fetuses and in non-dilated intermediary parts. Besides, the hormonal agents (estrogens) must be chiefly responsible for the hypertrophy, since, in a previous publication (1948, 4Fa), we have shown that estrone injected into castrated rats induced the growth of the nucleus to twice its size, which is equal to the nuclear volume found during the normal estrous cycle and during pregnancy.

That the real origin of the nuclear growth during pregnancy appears to be at best unknown, is illustrated by the fact that Keiffer (1928, '29) attributed uterine enlargement (human and guinea pig) to the imbibition of cellular water. The author also records that the increase in size to all muscle cells and the increase in number of myofibrillas is connected with the extensibility and contractibility of the tissues. He also holds these changes to be due to hormonal agents.

Our karyometric research however on endometrium and myometrium during the estrous cycle and estrus artificially induced by oestrone (1948, 1947a), as well as the results recorded in this paper, prove that the increase of the nuclear volume is not steady but rhythmic, the modal values of "leap" being in proportion to the basic value at the rate of 1:2:4:8 etc.

Modern karyometric research work on the interphasic growth of the nucleus shows a very evident relation between the nuclear volume and the number of chromosomes and chromonemata. Fabris (1935) already had said "It is interesting that it should be the nucleus and not the cytoplasm that starts hypertrophy." In 1935 this author put forward the following hypothesis: "It seems possible that the increase in size of the nucleus is related to a prolific cellular capacity."

From the numerous karyometric researches we may conclude that the *rhythmic growth of the nucleus* is related to the *multiplication of the nuclear "genome" or genetic elements* during the interphasic growth of the nucleus. Consequently, we must admit that we call hypertrophy is, at least as regards to the nucleus, due to multiplication of the nuclear genome.

This interphasic growth of the nucleus may show an intermediary value of 1.5 times the basic volume which has been discussed in many of Schreiber's researches and in a previous publication in collaboration with the author (1947a).

We found that on the 7th day of pregnancy (Figure 2) the greatest number of nuclei show twice their basic volume (mode III) and some show 1.5 times their basic volume, which points to a pause in interphasic growth. On the 13th and 14th day of pregnancy small nuclei are present, a phenomenon which we shall discuss later when dealing with hyperplasia. On the 20th and 21st day of gestation the

results obtained are very interesting, especially as to the myometrium, since, besides the absence of small nuclei, the histogram shows that several nuclei keep on growing and attain three, four and six times their primitive size (mode IV, V and VI).

In the endometric tissues the nuclei are large, but generally they do not exceed twice their primitive size (mode III).

Summarising, our karyometric researches on uterine tissues show that hypertrophy during pregnancy depends on *phenomena connected with the multiplying mechanism of the cells*. The real nature of hypertrophy of nuclei, is interphasic growth, both when mitotic division follows, and probably endomitotic growth when the nuclei exceed twice their primitive size without dividing. For a further discussion on the mechanism and significance of this rhythmic growth of the cell nucleus the reader must refer to Schreiber (1943, '46).

Hyperplasia: A considerable number of authors, among them Vilian (1849), Heschl (1852), Luska (1864), Veit (1885), Sanger (1887), Nagel (1896), Morales, Hoekl and Meyer all quoted by Froböse (1932), believe that hyperplasia is the principal factor of uterine growth. Amati (1992), quoted by Fabris (1935), observed hyperplasia in the myometrium of rabbits brought about by mitotic division of the muscle cells during the first days of pregnancy. Cattani and Pestalozza (quoted by Fabris, 1935) and Grynfeldt (1924-'25) found mitoses in the muscle cells of the human at their highest frequency during the first months of gestation. These facts were confirmed by Gander (1930) who observed hyperplasia in the uterine muscle of pregnant rats and mice resulting only from mitoses of pré-existent muscular cells. The same was held by Froböse (1932) who, noted in rabbits that "the muscle cells increased in number by mitotic division."

Fischer-Wassels and Büngeler (1931) found mitoses of the muscle cells in the uterus of baby mice after treatment with urine from pregnant woman, and Allen, Smith and Gardner (1937) observed the same in normal rats and mice treated with anterior pituitary extract and pregnant-mare serum. Crandall (1938) employing the colchicine method found abundant mitoses in the circular muscle of the uterus of rabbit, right at the beginning of pregnancy. Generally, authors admit that the generation of new cells in the uterus, both in the myometrium and endometrium, is limited to beginning of gestation, although Froböse observed that it may occur during the whole period of gestation.

Our own observation also shows, as may be seen on the histograms, that hyperplasia is present on the 13th and 14th day, when even the histologic picture is similar to that of estrus. The presence of small nuclei (mode I) besides the presence of numerous mitoses in the endometrium and rare mitoses in the myometrium (mitotic index 1.1 and 0.4%) indicate hyperplasia, since it is highly probable that the small nuclei are the product of division of large (mode III).

On the other hand, the fact that we find on the 13th and 14th day of pregnancy a picture similar to that of estrus confirms the experiments made by Swezy and Evans in 1930. These authors relate that the graafian follicles in rats show growth and regression at a rate approximately corresponding to the rhythm of the estrous cycle i.e. on the 5th, 10th, 14th and 18th day of gestation. It's quite possible that the values shown on our histograms of the 13th and 14th days of pregnancy of the rat, will also be found on the other days indicated by Swezi and Evans. Moreover, the histograms of the 7th day are very similar to those of the proestrus (Salvatore, 1948). If this is so,

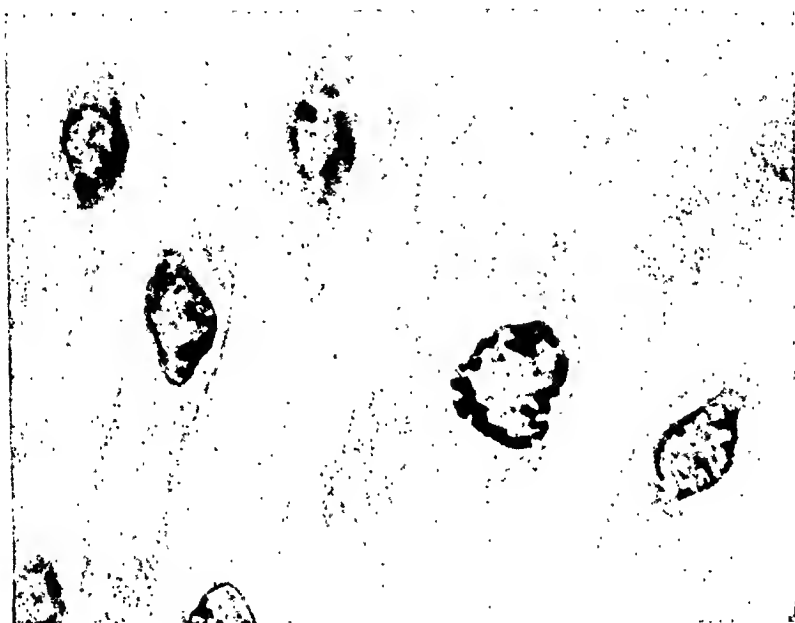


FIG. 4. Typical mitoses (prophase) of muscle cells on day 20 of pregnancy (magnification $\times 1480$).

mitoses will be found during the several stages of pregnancy (in rats) including the last days in the endometrium as well as in the myometrium. On Figure 4 are shown typical mitoses (prophase) of muscle cells on the 20th day of pregnancy.

Furthermore, the partial development of the follicle in the ovaries as well as the presence of mitotic forms in the uterus would be dependent on the threshold of the circulating hormone. In our foregoing publication (1948), we said that one fact struck us as very significant: after discontinuing the oestrone treatment of castrated animals there appeared numerous mitoses, which might indicate that the decline of the estrogenic threshold is the factor which determinates the mitoses in uterine tissues. Crandall holds however that the hyperplasia in the myometrium is due to a combined effect of estrin and progesterin.

There is, however, another factor that may determine the presence of hyperplasia: it is the mechanical distention of the uterus as proved by the experiments of Reynolds and Allen (1937), Reynolds and Kamminester (1937a, 37b) and Allen, Smith and Reynolds (1937) who employed the colchicine method, found typical mitoses in the muscular cells of castrated rabbits and in the castrated rabbits after progestin had been injected. It must, however, be kept in mind, that hyperplasia without hypertrophy—and vice versa—has never been found, not even in those objects of examinations obtained by mechanical distention, which proves the inter-relation of these two phenomena.

As was shown with reference to the estrous cycle and to artificially induced estrus in our previous publications (1948) and may be read off our present histograms, *hypertrophy* always precedes the appearance of mitotic forms, which proves that nuclear hypertrophy is actually the *interphasic growth of the nucleus*. Soon after this first phase, during physiological estrus, after discontinuing estrone treatment, and on the 13th and 14th day of gestation (very likely also in other stages), mitotic division sets in, the evidence for which, on our histograms, is the presence of small nuclei and mitotic forms. It is probable that during pregnancy only a part of the cells is subject to division, while the other either stops growing at twice their original volume or continues its interphasic growth, attaining to large volumes as is shown on the histograms corresponding to the 20th and 21st day of pregnancy.

The absence of small nuclei (mode I) on the 20th and 21st day of pregnancy, and the presence of such on the 13th and 14th day, together with the decrease in number of large nuclei (mode III) and accompanied by mitoses, seem to show that the new cells of the myometrium have their origin in pré-existent muscular cells of the uterus. In our opinion evidence for this, is to be found on the histograms corresponding to physiological and artificially induced estrus, completing our previous paper (1948).

On this point there is difference of opinion. Keiffer (1928, 1929) would have muscle cells originate from fibroblasts, Joachimovits (1928, 1929) from special forms of histiocytes. Stieve (1929a, 1929b, 1932) agrees more or less with both. Froböse (1935) contends for a combined original from connective tissue and muscle cells. Crandall (1938) and Corner (quoted by Reynolds, 1939) agree that a transformation of connective tissue to muscle cells is possible.

With these Fischer-Wassels and Büngeler (1931) disagree on the basis of their trypan blue, for muscle cells never contain granules of the dye as do connective tissue cells.

On the basis of the author's previous work on the estrous cycle of the rat and the present study of the pregnant rat, the most reasonable interpretation of the statistical findings with the karyometric tech-

nique employed is that new muscle cells of the uterus arise from pré-existing muscle cells.

CONCLUSIONS

The variations of nuclear volumes in the superficially and glandular endometrium and in the myometrium during pregnancy are similar and take place simultaneously according to the stage of pregnancy.

The increase in volume of the nuclei is exactly the same in the enlarged uterine parts, enclosing foetus, as in the non-dilated intermediary parts.

The increase in the nuclear volume during pregnancy is not steady, but rhythmic, i.e. it proceeds in leaps. The duplication of the nuclear size between 2 steps, indicates with probability the genetic nature of that growth.

The uterine growth is mainly consequence of one phenomenon, i.e. the multiplication of the nuclear genome. Hypertrophy and hyperplasia are manifestations of this process.

a) Hypertrophy of the nuclei is mainly interphasic growth, after which mitotic division take place; probably it may also be "endomitotic growth" when the nuclei keep growing and attain three, four and six times their primitive size even though they do not divide. The genetic significance of the rhythmic growth has been discussed in the papers of Schreiber.

b) Hyperplasia is the final of interphasic growth of several nuclei.

In the myometrium hyperplasia very likely takes place by means of division of pré-existent muscular cells and not by means of transformation of connective tissue cells into muscular cells.

On the 13th and 14th day of pregnancy the cells multiply in cycles similar to those that may be observed during estrus.

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THE USE OF STILBESTROL INHIBITED MALES AS TEST ANIMALS FOR GONADOTROPHIC HORMONES

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THAT ESTROGENIC substances have an inhibiting effect on the gonadotrophic activity of the pituitary of mammals has been amply demonstrated in endocrine literature. The use of stilbestrol as a "fattening" hormone in chickens has shown that a pronounced modification of the rate of function of the pituitary may be accomplished. Implants of as little as 15 mgs. of stilbestrol into males will cause complete regression of the testes and subsequently the comb, while larger doses will lead to a cessation of growth and will induce conditions in some instances resembling surgical hypophysectomy (Nalbandov & Card, 1943). These observations led to the idea that the physiological inhibition of the pituitary by estrogen may be used in the laboratory in place of surgical hypophysectomy. The advantage of the former lies in the fact that it is possible to make implants into large numbers of animals in a very short time, that mortality is as low as that of controls and that physiologically hypophysectomized males may be restored to normal, if that should be desired, by removing the pellets of stilbestrol. Unlike hypophysectomized animals, physiologically inhibited males can be kept under the same environmental conditions as untreated controls, the mortality being not any greater than that of the controls. It is also possible to produce "degrees" of physiological hypophysectomy by varying the dosage of estrogen given.

The disadvantages of this method are that occasionally a few of the males (1-2%) do not respond to the dose of stilbestrol given. In some males, a very small percentage, the implants become incapsulated and the pituitary may begin to function subnormally or even normally at an inopportune time in the experiment. Such cases are, as a rule, easily detected. In spite of these drawbacks, which are not any greater than the possibility of incomplete hypophysectomy or the danger from postoperative mortality, it appears that physiological hypophysectomy of males has definite advantages as a substitute for surgical hypophysectomy.

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ANIMALS AND TECHNIQUES

White male rats of the Sprague-Dawley strain weighing around 40 grams were used. In one experiment a pellet containing 15 mg. of diethylstilbestrol was implanted subcutaneously into each rat while in another experiment two such pellets were used. The implant can be made with or without light ether anesthesia, the slit in the skin being made in the region of the posterior dorsal third of the body. The pellet is then manipulated until it is lodged between the shoulder blades. It is recommended that reasonably clean techniques be used during this operation and that the pellet be dipped in alcohol prior to implantation. These precautions reduce the danger of infection and abscess formation which contribute to the undesirable incapsulation of pellets.

In the chicken the technique is similar except that here the implant is made in an area which is free from feather tracts, usually along the breast. This makes it possible to see the pellet through the skin and to check the birds periodically for the condition of the implant. In all our experiments, White Leghorn chickens were used. These are cheaper than are the males of the heavy breeds and are found to be much more sensitive to exogenous and endogenous hormones. The ages of the birds used varied from 20 to 200 days. In the experiments discussed here, the males weighed on the average 750 grams at the start of the experiment and were about 70 days old.

To test the response of stilbestrol implanted males to gonadotrophic hormones, PMS (Gonadogen) and purified preparations of LH and FSH were used. The PMS was injected at the rate of 25 Cartland-Nelson units per male in 8 days, while the FSH or LH preparations were injected at the rate of 5 grams equivalent (in terms of acetone dried sheep pituitary powder) per male in 8 days.

On all rats the following measurements were obtained at the time of autopsy: body weights, testes, seminal vesicles, adrenals, thyroids, and pituitary weights. In chickens, in addition to the above measurements comb size (the product of length and height) was used as the index of the rate of androgen secretion.

Two experiments using rats were done. In the first, the implants were made when the males were 21 days old. Each 7-day period after the day of implantation 5 treated and 4 untreated control rats were killed and the organ weights obtained. Thus a group of 5 males each was killed when it had been under the influence of stilbestrol for 7, 14, 21, and 28 days, respectively. The rats in each group were compared with untreated animals of the same sex and age.

In the second experiment two 15 mg. pellets of stilbestrol were implanted into each male and a period of 21 days was allowed to elapse before the first sample of organs was taken. At the end of the 21-day implantation period, some of the estrogenized rats were started on injections of pregnant mare serum (PMS—Gonadogen). Again in this experiment autopsy was performed every 7 days so that successive groups of 3 rats each had been implanted 21, 28, 35, 42, 49, and 56 days, respectively. Other groups (5 rats in each) had been implanted the same number of days as the above groups but in addition had been injected with PMS for 7, 14, 21, 28, and 35 days. A third series of groups of 3 rats each had received neither hormone treatment and served as normal controls.

TABLE 1. COMPARISON OF THE EFFECTS OF STILBESTROL ON THE ORGAN WEIGHTS OF RATS AND CHICKENS

Animals used	Length of experimental period (days)	Hormone Treatment	Testes			Seminal Vesicles or Comb Area				Average weight of organ during experimental period (% of body weight)		
			Start		Finish		Start		Finish			
			Weight (gms.)	% of body weight	Weight (gms.)	% of body weight	Weight (gms./cm. ²)	% of body weight	Weight (gms./cm. ²)	% of body weight	Testes	Seminal vesicles/comb
Male Chickens	35	Control Stilbestrol	0.22	0.02	2.33*	0.16	25.99	—	62.38*	—	0.10*	—
			0.21	0.02	0.48*	0.003	13.39	—	14.50*	—	0.01*	—
Male Rats	35	Control Stilbestrol (15 mgs.)	0.69	0.90	2.72*	1.34	0.66	0.07	0.45*	0.22	1.03*	1.20
			0.30	0.44	0.20*	0.18	0.07	0.09	0.13*	0.12	0.32*	0.10
Male Rats	50	Control	2.51	1.39	3.24*	1.98	0.18	0.10	1.16*	0.39	1.23*	0.24
		Stilbestrol (30 mgs.)	0.19	0.16	0.16*	0.12	0.09	0.08	0.13*	0.10	0.14*	0.09
		PMS + Stilbestrol	0.73	0.62	2.66*	1.61	0.17	0.15	1.78*	1.08	1.31*	0.72

* Differences significant at 1% level.

The experiments involving chickens also consisted of two phases. The first was planned to obtain information on the effect of prolonged action of stilbestrol on the physiology and the organ weights of males. In a second experiment the response of estrogenized cocks to FSH and LH was studied. The first experiment was set up in the same manner as was outlined for the rat trials, the last group of birds being killed 35 days after the implants were made. Three normal controls and 4 estrogenized chickens were killed at the end of each of the 7 day intervals.

RESULTS

Rats: The results obtained following stilbestrol inhibition are in agreement with the traditional expectations of the effects of estrogen on the pituitary gland.

Statistically significant (at the 1% level) reductions were obtained in body weights, testes and seminal vesicle weights (Table 1). The differences between the normal controls and the stilbestrol treated males with regard to adrenal, thyroid, and pituitary weights were statistically not significant, but in all cases these glands were heavier (in percent of body weight) in the inhibited males than they were in the controls.

The testes of estrogenized rats were reduced to about $\frac{1}{3}$ of the weight of the testes of the controls during the shorter experimental period and to about $\frac{1}{10}$ of normal during the longer inhibition period. In proportion to the body weight, the seminal vesicles were only slightly smaller in the experimental group during the shorter inhibition period. This difference, however, was much more pronounced after the more prolonged inhibition. On an actual-weight basis, the seminal vesicles did not stop increasing in weight in either group in spite of the complete arrest of testes growth. This was explained by the finding on histological study of these structures that large amounts of connective tissue were laid down presumably as the result of prolonged estrogen treatment. The epithelium of the seminal vesicles was, however, of the typical castrate appearance and the glandular portion of this structure was found by microscopic examination to be completely atrophic. Results of injection of PMS into stilbestrol inhibited males showed that, in spite of prolonged inhibition of the pituitary with estrogen both the testes and the seminal vesicles retained their sensitivity to their respective trophic hormones (Fig. 1).

The males injected with PMS did not show any difference in the rate of gain in body weight from their untreated controls but they continued to gain after the body weights of controls reached a plateau. The final body weight of the inhibited males treated with PMS was significantly higher than that of the controls. It is possible that this growth response is due, at least in part, to a thyrotrophic effect of PMS. In addition to this experiment, thyrotrophic action of PMS

was also noted in tests which involved baby chicks. Whether this effect is due to thyrotrophic action of PMS itself or whether it is due to the release of thyrotrophic hormone from the animal's own pituitary under the influence of PMS, remains problematic. It appears possible that the growth stimulating effect of PMS noted here and elsewhere may be mediated through the thyroid.

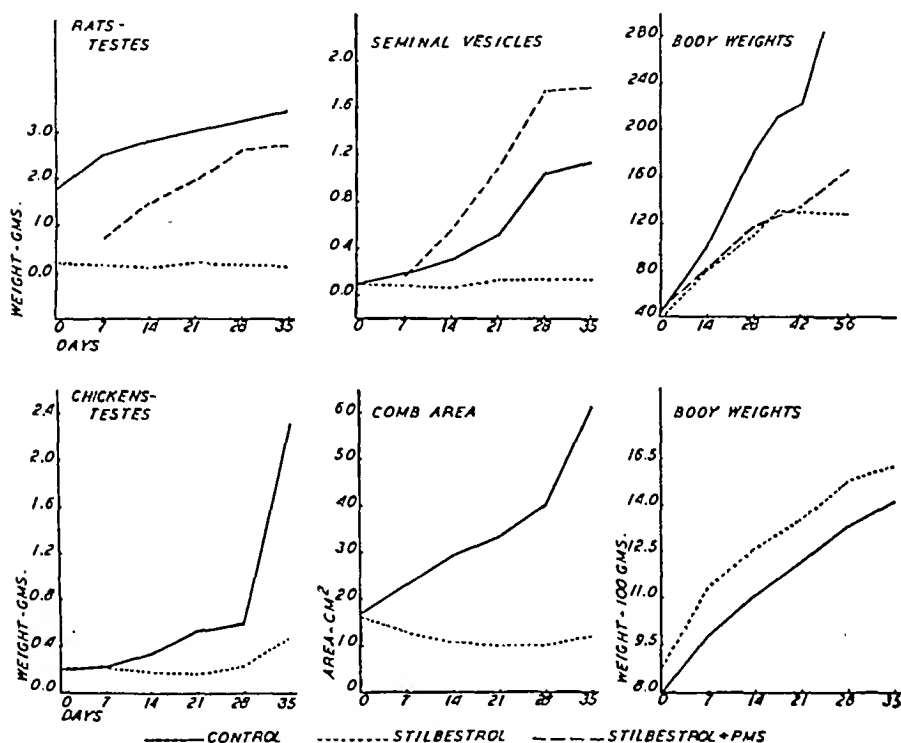


FIG. 1. Comparison of the effects of stilbestrol inhibition on body weights, comb areas, testes weights, and seminal vesicle weights of chickens and rats.

Chickens: In general, the same effects that were observed in the rat also held true for the chicken. The major difference between the responses of the two species lies in the fact that the dosage of stilbestrol used (15 mg. per animal) was not enough to cause any change in the rate of gain in the chicken. At the level given, stilbestrol led to unusual deposition of abdominal and subcutaneous fat, but the rates of gain for the treated group and their controls were not significantly different. The differences between the testes weights and the comb areas between the treated and untreated cocks were very great and statistically significant at the 1% level (Table 1). The differences in weight between the other organs measured were due to chance alone.

Stilbestrol inhibition reduced testes weights within two weeks after

the implant to 0.15% of body weight, while in the controls the testes weight was 0.31%. By the fifth week after the implant the inhibited testes were 0.03% of body weight while the controls had testes which comprised 1.64% of body weight. For the whole experimental period the average testes weights of the inhibited birds were 0.13% while those of the controls were 0.56% of body weight. The reduction in testes weight during the first 4 weeks of stilbestrol inhibition is not as great as that observed following hypophysectomy but is as pronounced at the fifth week as has been observed after surgical hypophysectomy.

Unlike the testes, the comb reaches its minimum size and levels off within 7 to 14 days after stilbestrol implantation (and incidentally also after hypophysectomy). In younger males and in those in which the comb is small, complete regression occurs within a minimum of 7 days, while this process requires a slightly longer time in older birds and in those in which the comb is developed beyond average. The rapidity of comb regression indicates that in both surgical and physiological removal of the pituitary, circulating gonadotrophic hormones, and hence androgen, reach a minimum level within a very short time. Evidence of nonsupport of the comb by androgen is apparent almost immediately (1 to 2 days) after either hypophysectomy or stilbestrol implantation. One of the main advantages of using physiologically or surgically hypophysectomized cocks lies in the fact that either incomplete inhibition of the pituitary or partial hypophysectomy become immediately obvious because of non-regression or continued growth of the comb.

EFFECTS OF GONADOTROPHIC HORMONES ON COCKS

In a previous paper Nalbandov, Meyer, and McShan (1946) have shown that hypophysectomized cocks are sensitive test animals for even traces of luteinizing hormone. It was shown that while preparations of FSH free from LH do not cause comb growth, they are capable of causing marked increases in testes size. Histological study of the testes confirmed the expectation that LH causes an increase in testes size by increasing the amount of interstitial tissue, while FSH has the same effect by its action on the proportion and size of the seminiferous tubules. Preparations of FSH contaminated with small amounts of LH were found to cause a significant increase in testes weight but no significant increase in comb area. Invariably, however, such a preparation did cause a reddening of the head region which is characterized in the untreated hypophysectomized bird by a grey pallor. Flushing of the skin of the head region, reddening and eventual growth of the comb are the result of the action on the testes of LH. The intensity and the degree of the response are directly correlated with the amount of LH injected or the ratio of this hormone to FSH if it is injected in a mixture with FSH.

Responses identical to those described for the surgically hypophysectomized cock were found in males inhibited with stilbestrol (Table 2). FSH alone can be distinguished from all preparations containing LH by the fact that the former never produces increases in testes weight of more than 60% over the controls while all LH preparations (at the levels injected) invariably caused testes weight increases of at least 100%. Pure FSH, FSH contaminated with LH, unfractionated anterior pituitaries, and pure LH produced the same effects on estrogen-inhibited males as they did on hypophysectomized males.

TABLE 2. COMPARISON OF THE EFFECTS OF DIFFERENT GONADOTROPHES ON THE ESTROGENIZED COCK

Hormone injected (after stilbestrol inhibition)	Color of head region and comb	Size of comb	Average testes weight (gms.)	Tubule diameter
None	pale grey	+	.28	+
FSH (pure)	pale grey	+	.39	++++
FSH and "trace" of LH	red	+ to +++	.60	++++
Whole sheep pituitary	red	++++	.64	++++
LH (pure ?)	red	++++	1.00	+ (or +++ ?)
PMS	red	++++	.67	++++
Untreated	red	normal	9.45	normal

In both hypophysectomized and estrogenized cocks maximum comb size following treatment with LH-containing gonadotrophic hormones was reached within 9 to 15 days after the first injection. For reasons which are to be discussed in a subsequent paper the comb size, and to some extent the testicle size, then decreased in spite of continued injection. Within about 10 days after reaching a maximum area, the comb returned to its preinjection size and became completely atrophic, while the testes leveled off or were reduced in size somewhat. Estrogenized or hypophysectomized males which have been treated once with an LH containing gonadotrophic hormone never again responded to such treatment, precluding the possibility of using such males more than once. This peculiarity is restricted as far as known to the chicken (possibly birds in general), and may not hold true for the rat although the evidence on this point is not complete.

THE USE OF COCKS AS TEST ANIMALS FOR GONADOTROPHIC HORMONES

The results discussed in the previous section make it possible to make the following recommendations for the use of males inhibited with stilbestrol as test animals for gonadotrophic hormones. The male rat appears to be as sensitive as is the chicken. The chicken has the advantage over the rat of having an externally visible and measurable secondary sex character, the comb, which is very sensitive to

even small doses of LH and hence androgen. It is also much cheaper than the rat and in many instances more easily available.

White Leghorn males were used because of their great sensitivity to hormones, their small size and the fact that they are cheap, occasionally being given away by hatcheries. They can be used at any age from 30 to 200 days of age. A 15 mg. pellet of stilbestrol is implanted subcutaneously and at the same time a measurement of the height and length of the comb is made. Further comb measurements are made on days 7, 9, 11, and 13 after the treatment. With younger birds the comb usually reaches its smallest size on day 7, and as soon as a plateau is reached injections are begun. Satisfactory tests are obtained with either 8 injections in 4 days or 8 or 16 injections in 8 days. A final comb measurement is obtained after the last injection, the chickens are killed and the testes removed for weighing. The stilbestrol pellet can be recovered and used over again although it is suggested that at least two "used" pellets be implanted to assure adequate inhibition. As already mentioned, estrogen inhibited or hypophysectomized birds once completely stimulated with an LH-containing hormone preparation lose their ability to respond to such mixtures and for that reason can not be used for the testes more than once.

DISCUSSION

While estrogenized male rats were found useful as test animals for gonadotrophic hormones, estrogenized cocks are preferred for this purpose in this laboratory. In both the rat and the cock sufficiently complete physiological hypophysectomy can be produced with stilbestrol to permit them to be used as test animals for gonadotrophic hormones. Estrogenized cocks appear better suited for qualitative and quantitative gonadotrophic hormone assays because they possess the externally visible and measurable secondary sex character, the comb, which was found to be a reliable and sensitive indicator for endogenous androgen. Furthermore the coloration of the head region was found to be a sensitive indicator for traces of luteotrophic hormone, such traces causing a reddening of the head skin. The obvious advantage of estrogenized over hypophysectomized animals lies in the ease of their preparation.

Estrogen inhibition has been tried on chickens of many age ranges and chicks as young as 30 days were found as useful as were older birds. In general, animals less than 6 months of age were found more sensitive to test hormones and easier to inhibit with stilbestrol than older birds. Injected gonadotrophic hormones cause more uniform increases in the testes of younger birds than they do in those of older inhibited males.

The estrogenized chicken is peculiarly well suited as a test animal for the qualitative and quantitative characterization of gonadotrophic hormones. Its use makes it easy to distinguish between pure FSH and

preparations of FSH contaminated with even small amounts of LH. The test is based on the "all or none" response of the comb and, provided the inhibition of the pituitary is complete, is a more reliable indicator of LH than are the ovaries of the immature hypophysectomized or intact rat. By taking into consideration comb size, testes size and the histological appearance of the testes, it is possible to determine whether an LH preparation is contaminated with small or large amounts of FSH. Although the data on this point are not sufficient, indications are that if a preparation of LH is completely free from FSH, it will not cause any increase in tubule diameter over that found in uninjected controls.

Because of the nature of the test and its reliability, the numbers of animals necessary for the characterization of a gonadotrophic preparation are smaller than would be required if female rats were used.

SUMMARY

In immature male rats and chickens the subcutaneous implantation of 15 mg. of diethylstilbestrol causes a complete inhibition of the animal's own pituitary. This in turn leads to a complete regression of the testes and atrophy of the secondary sex organs supported by androgen.

This effect makes it possible to use such estrogenized males as test animals for the characterization of gonadotrophic hormones. This substitute for surgical hypophysectomy has many obvious advantages over the more time consuming technique.

Estrogenized immature cocks were found to be particularly well adapted to serve as test animals for quantitative and qualitative assay of gonadotrophic preparations. By considering the changes produced in comb size, testes weight, and testes histology it is possible to distinguish positively between pure FSH, FSH and LH mixtures, and pure LH preparations, and naturally occurring mixtures of the two hormones.

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FORMATION OF SECONDARY DECIDUOMATA IN SPAYED MICE AND LACTATING RATS

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INTRODUCTION

IT HAS BEEN known since the classical observation of Loeb (1907) that the properly sensitized uterine endometrium responds to a traumatic stimulus with a rapid local proliferation that imitates the maternal portion of a placenta.

This growth has been named a deciduoma or placentoma and it is mainly during the last decade that our knowledge about its morphology and experimental physiology has been considerably advanced as a result of the work of Selye and McKeown (1935), Krehbiel (1937), Rothchild et al. (1940), Astwood (1939), Atkinson (1944) and others.

We now know that a deciduoma develops only under highly specific hormonal conditions, that it inevitably and rapidly involutes 5-6 days after its coming into existence, that its life span cannot be appreciably prolonged beyond that time and that a "metrial gland" (another short-lived structure) develops when the deciduoma starts to regress, only to completely disappear after 5 more days.

However, a number of important questions have remained unsettled. One of these has been the subject of a controversy in recent years: it has been repeatedly affirmed and denied that a (primary) deciduoma, once established, inhibits the development of a subsequent (secondary) deciduoma.

The earliest relevant statement came from Selye and McKeown (1935) who, in the course of other extensive studies on the experimental physiology of deciduomata, observed that in a series of 11 lactating rats, secondary deciduomata developed in only six instances.

Subsequently, Rothchild and Meyer (1942) stated without data that primary deciduomata do not inhibit secondary deciduomata in the spayed rat.

Next, Lyon and Allen (1943), confirmed Selye and McKeown's original observation in lactating rats, and as they were able to overcome the inhibition with estrogens, suggested that the growing primary deciduoma might consume estrogen.

Two years later, Atkinson and Leathem (1945) reported a complete inhibition of secondary by primary deciduomata in the spayed

mouse but did not speculate on the nature of the mechanism.

It was only recently that the problem was approached with more quantitative methods. Peckham and Greene (1947) studied the inhibition in spayed rats and lactating rats with adjusted litters, applying four stimuli per uterine horn and thus making it possible to assay the intensity of the response. They also measured the size of each deciduoma (maximal diameter of the unfixed tissue), calculated statistical significance of results and checked for the non-specific effect of surgical trauma by performing mock-operations in place of the primary stimulation in control groups. They found no significant depression in either the number or the size of secondary deciduomata in the lactating rat. In the spayed rat they observed a significant depression in the size, not in the number, of secondary deciduomata timed similarly and preceded by a mock-operation instead of a primary stimulation. Hence they concluded that the depression of the size was due to the non-specific effect of surgical trauma and prolonged injections.

In summary then, the pertinent literature suggests that there is no specific inhibition in the spayed rat, that it exists in the spayed mouse, that it is doubtful in the lactating rat and that whatever inhibition is observed is due to non-specific effects.

As the whole question has possible implications in the fields of anti-tumorigenesis, implantation physiology and stress pathology, it was the purpose of this study to investigate the following points:

1. Does the inhibition exist in the spayed mouse or the lactating rat?
2. Is the time interval between the two stimuli essential for the inhibition, i.e. is the metrial gland involved?
3. Do non-specific factors depress the deciduoma response?

MATERIALS AND METHODS

Female Wistar albino rats weighing 250-300 gm. were used for the lactation experiment and female Swiss albino mice weighing 25-30 gm. for all other experiments. The mice received a daily subcutaneous injection of 1 mg. progesterone in 0.05 ml. maize oil for varying periods of time after spaying. The uterine trauma consisted in passing a #50 black cotton thread transversely through the uterus and tying it loosely. In almost all instances three threadings were applied per horn. Experimental and control groups were run simultaneously. The deciduomata were assayed 48 hours after fixation of the uteri in 10% formalin. The evaluation of the response was based on three criteria:

1. *The number of deciduomata developing per horn.* We preferred to express it as mean response per horn instead of pooling all positive responses in a group and expressing them as a percentage of the total possible number of responses. In this way we were able to calculate standard errors of the means and avoided the erroneous comparison of percentages between unequal groups.

It should be noted that the counting of macroscopically unquestionable deciduomata alone frequently misses small but microscopically definite reactions. It sometimes happened that after histologic examination the number of positive responses was found to be considerably higher than before. Step-serial sections were therefore done in every macroscopically negative or doubtful instance.

2. *The maximal diameter of the deciduoma* measured at an axis perpendicular to the mesometrial-antimesometrial axis. All measurements were made by the same observer to the nearest 1/64 of an inch and expressed in this unit. But the diameter does not always reflect the real size of the deciduoma. An oval or oblong deciduoma may have a greater total volume than a spherical one of the same diameter.

3. *The mass of the deciduoma-bearing horn in milligrams.* This third criterion was introduced in order to get a direct expression of the quantity of the newly proliferated deciduoma tissue. It should be noted that the range of weight of a non-stimulated horn under progesterone is 15–35 mg., with a mean of 22. After stimulation, the horn may weigh from 100 to 350 mg. depending on the stage of the deciduoma development. In our opinion, the weight was the most reliable index of the intensity of the response.

The standard error (ϵ) of any mean was calculated from the formula

$$\sqrt{\frac{\Sigma d^2}{n^2 - n}};$$

the standard error of the difference between two means from the formula

$$\sqrt{\epsilon_1^2 + \epsilon_2^2};$$

the probability that the difference between two means is due to chance (P) from Fischer's table of t . Any difference with a P greater than 0.01 was considered not significant.

RESULTS

(A) *Spayed Mice.* The experiments with spayed mice can be classified in the following five groups:

a) *Heterolateral application of primary and secondary stimulus with variation of the time interval between the two stimuli.*

These experiments were done in order to find out whether the reported desensitization of the contralateral horn by a primary deciduoma was a function of the time that separated the two stimulations. Assuming that a specific inhibition existed, there were reasons to expect that at a certain time it would be maximal, while later on it would disappear.

For this purpose various groups of mice were spayed and beginning 48 hours later injected with 1 mg. progesterone daily until they were killed. On the 4th day after commencement of the progesterone treatment the left horn was stimulated in all experimental groups, while a laparotomy was simultaneously performed in all controls. The right horn was stimulated in both experimental and control

groups at different intervals (3, 6, 8 and 11 days) after stimulation of the left horn, a pair of experimental and control groups being dedicated to each interval. All animals were killed four days after stimulation of the right horn. The characteristics of the right horn deciduomata as well as the mean number of the left horn responses are contained in Table I. (The experiment with an interval of 3 days between the two stimulations was performed twice.)

TABLE I

Time interval between the two stimulations	Group	Number of animals	Mean number of decid. per right horn $\pm \epsilon$	Mean diameter of right horn decid. $\pm \epsilon$ (in 1/64")	Mean weight of right horn decid. $\pm \epsilon$ (in mg.)	Mean number of decid. per left horn $\pm \epsilon$
3 days. First perform.	Exp.	10	2.6 ± 0.3	6.5 ± 0.3	110 ± 12	2.8 ± 0.1
	Control	7	3.0 ± 0	6.3 ± 0.1	97 ± 4	
	P		0.2	0.6	0.2	
3 days. Repetition	Exp.	13	2.8 ± 0.15	6.7 ± 0.8	118 ± 13	2.7 ± 0.2
	Control	13	2.7 ± 0.2	6.1 ± 0.6	105 ± 11	
	P		0.6	0.6-0.5	0.5	
6 days.	Exp.	8	2.5 ± 0.3	6.3 ± 0.4	103 ± 12	2.8 ± 0.2
	Control	10	1.9 ± 0.4	5.2 ± 0.5	69 ± 9	
	P		0.3-0.2	0.1	0.05	
8 days.	Exp.	9	2.1 ± 0.4	6.4 ± 0.7	119 ± 31	2.7 ± 0.1
	Control	6	2.6 ± 0.4	6.5 ± 0.6	114 ± 49	
	P		0.6	0.9	0.9	
11 days.	Exp.	7	2 ± 0.3	4.8 ± 0.3	58 ± 7	2.6 ± 0.2
	Control	7	1.9 ± 0.3	4.9 ± 0.6	64 ± 11	
	P		0.2	0.9	0.7	

The results clearly indicate that there is no significant difference between the number, size or mass of the deciduomata that were preceded by other deciduomata and those that were preceded by a laparotomy, whether the two stimulations were 3, 6, 8 or 11 days apart. One is driven to the same conclusion if the mean values of all experimental and all control groups are pooled as in Table II.

It is striking that the deciduomata that developed after 16 days of progesterone treatment (the 11 days interval between the two stimuli)—whether preceded or not by other deciduomata—were significantly smaller in size and mass than those of every other single group except the control group in the 6 days interval. Due to its

TABLE II

Group	Number of animals per group	Mean number of decid. per right horn $\pm \epsilon$	Mean diameter of right horn decid. $\pm \epsilon$ (in 1/64")	Mean weight of right horn decid. $\pm \epsilon$ (in mg.)	Mean number of decid. per left horn $\pm \epsilon$
Experimental	47	2.4 \pm 0.14	6.1 \pm 0.42	101 \pm 11	2.7 \pm 0.1
Control	43	2.4 \pm 0.25	5.8 \pm 0.3	90 \pm 9	
P		0	0.6	0.5	

singular occurrence, the latter irregularity must be attributed to chance.

b) *Effect of extirpation of the primary-deciduoma-bearing horn on secondary deciduomata.*

One would expect that if a primary deciduoma exerts any influence on a secondary, its extirpation prior to the second stimulation should remove that influence. For that purpose eight mice were spayed, and beginning 48 hours later injected daily with 1 mg. progesterone until they were killed. On the 4th day after commencement of the injections the left horn was stimulated. On the 8th day, this horn with its fully developed deciduomata was extirpated and on the 11th day, the right horn was stimulated. The animals were killed on the 15th day.

A control group was run simultaneously, timed similarly and subjected to the same experimental procedure, except for the fact that the extirpation of the left horn deciduomata was omitted.

The right horn deciduomata of both groups are compared in Table III.

TABLE III

Group	Number of animals per group	Mean number of decid. per right horn $\pm \epsilon$	Mean diameter of right horn decid. $\pm \epsilon$ (in 1/64")	Mean weight of right horn decid. $\pm \epsilon$ (in mg.)	Mean number of decid. per left horn $\pm \epsilon$
Hysterectomized	8	2.1 \pm 0.4	5.2 \pm 0.4	102 \pm 20	3 \pm 0
Intact	8	2.5 \pm 0.3	6.3 \pm 0.4	103 \pm 12	2.8 \pm 0.2
P		0.4	0.1-0.05	0.9	

It can be seen that the removal of the primary deciduomata did not affect the secondary deciduomata.

That the insignificant depression of the average values for number and size of secondary deciduomata in the hysterectomized group was not due to the greater surgical trauma in that group was further proven in subsequent experiments designed to study the effect of non-specific stress on deciduomata.

c) *Homolateral application of primary and secondary stimulation.*

If a deciduoma inhibits another deciduoma by either consuming or producing a hormone, the resulting gradient of inhibition should have its maximum in the uterine area immediately adjacent to the primary deciduoma. To test this, six mice were castrated and beginning 48 hours later, received daily injections of 1 mg. progesterone until they were killed. On the 4th day of progesterone treatment the left horn was stimulated at two points separated by a distance of about one cm. On the 11th day, a single stimulus was applied between the two deciduomata that had developed in the left horn as a result of the stimulation on the 4th day. The right horn was also stimulated singly on that occasion. Three days later the animals were killed. The secondary deciduomata of the left and right horn are compared with each other in Table IV.

TABLE IV

Group	Number of animals	Total number of secondary decid.	Expected number of secondary decid.	Mean diameter of secondary decid. $\pm e$ (in 1/64")	Total number of primary decid.	Expected number of primary decid.
Secondary decid. of the left horn (=homolateral to primary decid.)	6	6	6	6 ± 0.3	11	12
Secondary decid. of the right horn (=contralateral to primary decid.)	6	6	6	6 ± 0.3		

It is evident from the data that there is no difference between the number or size of secondary deciduomata developing in the horn containing the primary deciduoma and those of the contralateral horn.

d) *Effect of non-specific stress on deciduomata.*

Peckham and Greene (1947) concluded from some of their experiments that the non-specific effect of surgical trauma and prolonged treatment was responsible for the significant depression of the secondary response in spayed rats and also suggested that the same non-specific factors "might have a greater influence on the (deciduoma) reaction in the mouse, i.e., instead of merely decreasing the size of the secondary deciduomata . . . they might prevent their formation."

The following experiment was designed to test this hypothesis: Four groups of spayed mice were treated with progesterone in the usual way, traumatized in the right horn on the 4th day and killed on the 8th day of treatment. Of these four groups one served as non-stressed control, while each of the other three was exposed to a different type of general stress during the entire period of progesterone treatment.

Cold, formalin injections and extensive operative trauma with repeated narcoses were the types of stress employed.

In the group exposed to cold the animals were put in the refriger-

ator at 0°C twice daily for 5 hours with an hour's rest period between the two expositions.

In the formalin treated group the animals were injected twice daily with 0.05 ml. of a 10% formalin solution subcutaneously.

The third experimental group was unilaterally nephrectomized, splenectomized and partially pancreatectomized on the second day of progesterone treatment and subjected to deep ether anesthesia twice daily for ten minutes during all subsequent days.

The characteristics of the deciduomata that developed in those four groups are compared with each other in Table V.

TABLE V

Group	Number of animals	Mean number of decid. per horn $\pm \epsilon$	Mean diameter of decid. per horn $\pm \epsilon$ (in 1/64")	Mean weight of decid. per horn $\pm \epsilon$ (in mg.)
I. Non-stressed control	13	2.7 \pm 0.2	6.1 \pm 0.6	105 \pm 11
II. Operations + anesthesia	6	3 \pm 0	7 \pm 0.5	122 \pm 19
III. Cold	8	3 \pm 0	7.4 \pm 0.35	119 \pm 18
IV. Formalin*	4	2.5 \pm 0.5	6.3 \pm 1.2	99 \pm 27
P of I versus II		0.2-0.1	0.3	0.5
P of I versus III		0.2-0.1	0.2-0.1	0.5

* The P of I versus IV was not calculated as only 4 animals survived in the formalin group (from an original number of 12). It seems certain however that formalin did not depress the deciduoma formation any more than the two other types of stress.

It is obvious that considerable non-specific stress applied continuously before, during and after the uterine stimulation does not depress the deciduoma formation in the spayed mouse. The higher average values for number, size and mass of the deciduomata in stress groups II and III, although not significant and probably due to the smaller number of animals in those groups, suggested the possibility that stress itself might synergize progesterone in the target tissue either through discharge of a luteoid hormone from the adrenals or through some other mechanism. It would be interesting to see whether stress alone, without progesterone, could sensitize the uterus. For that reason four mice were spayed; starting 48 hours later they were put in a refrigerator at 0°C for ten hours daily and exercised in the revolving chamber for two hours daily until they were killed. On the 4th day after commencement of stress the uterus was stimulated in the standard way and on the 8th day the animals were killed. Another 4 animals served as non-stressed controls; of these, 2 received progesterone and 2 did not.

No deciduomata developed in any of the experimental animals.

Histologic examination of the uteri revealed that by Hooker's criterion (1945) there were no demonstrable luteoid or folliculoid hormone-levels in the organism. The control animals reacted as expected. (B) *Lactating rats*. In the experiment of Peckham and Greene (1947) with lactating rats the second stimulus was applied 3 days after the first one. As earlier investigators who obtained a depression of the secondary response had applied the second stimulus later, at a time when a "metrial gland" had formed as a result of the first stimulation, it was considered appropriate to separate the two stimuli by an interval of 6 days.

In an experimental group of seven lactating rats the left horn was traumatized on the fifth day and the right horn on the 12th day after parturition. The animals were killed on the 16th day.

In another control group of seven lactating rats, timing and operations were identical except for the fact that a mock-operation was performed in place of the stimulation of the left horn. The litters were adjusted to 7 ± 1 sucklings per lactating animal in both groups, throughout the experiment.

The characteristics of the deciduomata resulting in both groups from stimulation on the 12th day after parturition are tabulated in Table VI.

TABLE VI

Group	Number of animals per group	Mean number of decid. per right horn $\pm \epsilon$	Mean diameter of right horn deciduomata $\pm \epsilon$ (in 1/64")	Mean weight of right horn decid. $\pm \epsilon$ (in mg.)	Mean number of decid. per left horn $\pm \epsilon$
Experimental	7	2.4 ± 0.4	9 ± 1	339 ± 64	2.7 ± 0.2
Control	7	3 ± 0	9.4 ± 0.5	413 ± 59	
P		0.2	0.7	0.5	

The results suggest that there is no significant difference between the number, size or mass of deciduomata that were preceded by other deciduomata and those that followed a mere laparotomy.

CONCLUSIONS

(A) *Spayed Mice*. It seems justified to conclude that the presence in a uterine horn of a deciduoma or a "metrial gland" at any stage of development does not hinder the development of another deciduoma in the same or the contralateral horn.

Furthermore, it appears that the deciduoma response is neither increased nor decreased by acute, non-specific damage to the organism, possibly because the latter fails to cause the discharge of threshold quantities of luteoid or folliculoid hormones from the adrenals. It is remarkable that even a stress so severe as to kill 75% of the

animals (formalin injections) did not influence the deciduoma response in the survivors.

Finally, there was a tendency towards a decrease of the mean number of responses per horn with increasing length of progesterone pretreatment; stimulation after the longest progesterone treatment (15 days), whether preceded or not by other deciduomata, resulted in responses that were markedly and significantly weaker than after almost every shorter treatment (cf. Table I). This suggests that long lasting progesterone levels diminish the deciduoma response. We seem to deal here with another instance of target refractoriness developing after protracted action of a steroid hormone.

(B) *Lactating Rat*. We could confirm Peckham and Greene's conclusions that there is no inhibition of secondary by primary deciduomata in the lactating rat.

The difference between our own conclusions and those of some earlier investigators is probably due to the great individual variation of the deciduoma reaction and to the difficulty of its quantitative assessment in unequal groups, on the basis of macroscopical counts alone, without further criteria or statistical evaluation of data.

SUMMARY

The formation of a deciduoma in one uterine horn of a spayed mouse is not influenced by the pre-existence in either horn of another deciduoma or metrial gland at any stage of development.

Acute non-specific stress does not affect the deciduoma response in the spayed mouse, nor does it cause the secretion of demonstrable luteoid or folliculoid hormones from the adrenals.

Protracted action of progesterone diminishes the deciduoma response in the spayed mouse.

Peckham and Greene's observations in the lactating rat are confirmed.

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PROGESTERONE AND ESTROGEN IN THE EXPERIMENTAL CONTROL OF OVULATION TIME AND OTHER FEATURES OF THE ESTROUS CYCLE IN THE RAT

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INTRODUCTION

SEVERAL YEARS ago it was reported that small daily doses of progesterone given to persistent-estrous rats serve to interrupt the equilibrium between hypophysis and ovaries, inducing sequences of vaginal cycles which closely resemble those in normal animals (Everett, 1940). In a significantly high proportion of these cycles ovulation occurs and new corpora lutea are formed. This finding cannot be explained on the assumption which has been generally held for many years that the principal effect of progesterone upon the hypophysis is suppression of LH secretion. Subsequent studies have made it more and more apparent that under certain conditions progesterone facilitates the release of luteinizer.

In 1943 we reported that if persistent estrus is interrupted experimentally by a single injection of progesterone, a diestrous interval of 2 to 3 days usually occurs followed by the typical proestrous smear picture and then full cornification which continues if no further treatment is given. Newly grown follicles persist under these conditions. If, however, a second injection of progesterone is given during late diestrus or early estrus, luteinization is usually induced and the cycle is completed. Sequences of such cycles can thus be maintained by an injection of 0.5 to 1.0 mg. of progesterone during proestrus of each cycle. More recently we have extended the investigation to normal rats. In a preliminary account (Everett, 1944) it was reported that in normal rats which exhibit predictably regular 5-day cycles the injection of 1.0 mg. of progesterone about noon on the third day of diestrus serves to advance ovulation time approximately 24 hours. Ovulation occurs during the night following injection and, of equal significance, the vaginal smears are usually cornified the next morning. On the other hand, no advance of ovulation time nor hastening of vaginal changes is obtained in 4-day cyclic rats injected 24 hours before the expected proestrus. This particular observation makes it especially evident that other factors intrinsic in the cycle are of primary impor-

tance and that the action of progesterone is secondary to these. In this connection it should be recalled that, during diestrous intervals experimentally induced in persistent-estrous rats, a single injection of progesterone early in diestrus is ineffectual in causing completion of the ensuing cycle (Everett, 1943), while it is most effectual if given near the onset of heat when estrogen levels are undoubtedly rising or already maximal.

Since the initial experiments of Hohlweg (1934) it has been repeatedly indicated that estrogen itself causes liberation of luteinizing hormone¹ from the rat hypophysis (Lane, 1935; Fevold, Hisaw and Greep, 1936; Hohlweg and Chamorro, 1937; Westman and Jacobsohn, 1938). It was also recently demonstrated in the pregnant or pseudopregnant rat that injection of a few micrograms of estradiol benzoate causes ovulation about 36–40 hours afterward (Everett, 1947). The presence of active corpora lutea thus appears quite compatible with LH liberation under some circumstances.² Yet under other circumstances progesterone seems to prevent LH release. Selye, Browne and Collip (1936) reported that daily injection of 4 mg. of progesterone in rats suppresses vaginal cycles. Phillips (1937) later demonstrated that daily injection of as little as 1.5 mg. will postpone indefinitely the next expected estrus if the treatment is begun during early diestrus. Dempsey (1937) noted in the guinea pig that daily injection of progesterone specifically prevents the advanced stages of follicle maturation. This was regarded as evidence for suppression of LH secretion. Using the augmentation reaction in rats treated daily with progesterone, Astwood and Fevold (1939) reached a similar conclusion. Makepeace, Weinstein and Friedman (1937) had found in the rabbit, meanwhile, that injection of progesterone for 5 days prevents ovulation after mating on the sixth day. Subsequently, Friedman (1941) reported that treatment of estrous rabbits for 24 to 72 hours greatly reduces the number ovulating in response to copper acetate injection.

The experimental results recorded below will, it is hoped, partially resolve the apparent disagreement between these results and our evidence that progesterone facilitates LH release. Preliminary accounts of certain of the observations reported here have been presented elsewhere (Everett, 1944, 1946).

MATERIALS AND METHODS

In the design of the several experiments described below a high degree of predictability was essential, with respect to cycle length, ovulation time and

¹ Or total gonadotrophin (FSH+LH). The expression LH is freely used in this paper in the conventional sense (cf. Hisaw, 1947), with full realization that FSH also enters into preovulatory stimulation of follicles. The phenomena with which we are dealing here may largely express quantitative rather than qualitative differences in gonadotrophin secretion.

² A more crucial demonstration of this point appears later in this paper.

other chronologic features of the estrous cycle. To this end, lighting is automatically controlled by a time switch which gives the colony 14 hours of light and 10 hours of darkness daily throughout the year. Room temperature is not controlled, but it has been our experience that only sudden weather changes produce large-scale changes in the cycles. We have been able to recognize such general effects in the colony as a whole by virtue of the fact that vaginal smears are taken daily in a group of 50 to 75 rats, only a small number of which are being treated experimentally at any one time. The remainder thus serve as controls for the experimental group.

We have consistently worked with inbred strains: the "normal" Vanderbilt (Osborne-Mendel) strain, and, for specific purposes, the defective DA strain. Long experience with the former has enabled us, after following the vaginal smear sequences for 2 to 4 weeks, to predict with rare failure the night during which ovulation will occur in a given animal having a consistent history of 4-day or of 5-day cycles. Two substrains of the Vanderbilt stock have been developed. In one of these (designated Va) most of the females have regular 4-day cycles after attaining full maturity. Frequently the first few cycles are 5 days long, but these usually give way eventually to 4-day cycles. From our records we have calculated that if a Va animal has experienced two 4-day cycles in sequence the probability is about 93% that the next cycle will also be 4 days in length. In the second Vanderbilt substrain (designated Ve) nearly all females have regular 5-day cycles. In this group, after two successive 5-day cycles have been observed in a given rat, the probability is 90% that the next cycle will also be 5 days long. There is only a 4% chance that this next cycle will be spontaneously shortened to 4 days and even this small chance may be nearly eliminated by selection of animals showing 3 days of clearly diestrous smears in the third cycle. In the defective DA strain, young virgin females are usually regularly cyclic during the third and fourth months of age, these cycles being mostly of 5 days' duration. Subsequently such animals become persistent-estrous (Everett, 1939, 1942). This condition is well established in nearly all cases before the age of 200 days. In certain instances specified below, first and second generation hybrids between the Vanderbilt and DA strains were employed. These were vigorous animals and most of them proved to have either 4-day or 5-day cycles which were highly predictable.

The vaginal smears are obtained daily at 8 to 10 A.M. by saline lavage, fixed in alcohol after drying and stained with 1% alkaline toluidine blue. The typical daily smear sequence thus found in 4-day cyclic Vanderbilt rats is as follows: *day 1*—(diestrus) heavily leucocytic with occasional remnants of clumps of cornified cells; *day 2*—(diestrus) moderately leucocytic; *day 3*—(early proestrus) absence of leucocytes, numerous small, nucleated epithelial cells among thin, non-nucleated squames (some animals tend to show a few leucocytes among the epithelial cells at this time, becoming clearly proestrous later in the day); *day 4*—(estrus) full cornification. Ovulation occurs during the night preceding day 4. The smear sequences in 5-day cyclic Vanderbilt rats are of two general types: In the first type (A) leucocytic smears are obtained on 3 successive days. On day 4 (proestrus) the smears are free of leucocytes and rich in small nucleated epithelial cells, usually in clumps. On day 5 (estrus) they are fully cornified, occasionally with beginning admixture of leucocytes and nucleated squamous epithelial cells. In the second type (B)

the smear on day 3 is free of leucocytes or nearly so and closely resembles the proestrous smear seen on day 3 of the 4-day cycle. On the next morning the smear is either of the late proestrous type, containing a mixture of small non-nucleated squames and round cells with indistinct nuclei, or slightly more advanced, containing a uniform spread of small cornified cells. On day 5 full cornification is seen as in the first type. Almost needless to say, intergrades between types A and B occur. In either type, ovulation occurs during the night preceding day 5. In the DA strain both types of 5-day cycle are encountered, but more commonly type A.

In predicting the night of ovulation we by no means employ as principal criterion the vaginal smear picture on the preceding day. Rather, the full sequence of smears over several cycles is considered and the events in the current cycle are predicted against this background, using the current smears to indicate any significant irregularities.

Actual ovulation time has been estimated in 4-day cyclic Vanderbilt rats by killing 28 of them between 12:45 and 4:00 A.M. during the night following proestrus. Preovulatory swelling or ruptured follicles were observed in all cases. Ovulation in progress was found as early as 1:10 and as late as 1:55. Completed ovulation (8 or more tubal ova) was found as early as 1:45 and in every case examined after 2:30. Although no such direct information has been obtained in 5-day cyclic rats of either strain, the histological appearance of new corpora lutea obtained from them during the late morning or early afternoon of day 5 is similar to that seen at such times on day 4 of the 4-day cycle.

In our routine procedure, direct visualization of tubal ova is accomplished after killing the animals with illuminating gas, by excising the ampullae and mounting them in physiological saline under a cover slip (Everett, 1947). In the rat, as in the mouse (Burdick and Whitney, 1941), the thin walls of the dilated ampullae allow ready identification of granulosa cell masses and ova during the first day after ovulation. The excised ovaries are examined in saline under dissecting lenses for the tentative identification of newly ruptured follicles.

In most instances described here the ovaries were fixed in Zenker's fluid, embedded in paraffin and serially sectioned at 8 to 10 μ . The routine stain was Harris' hematoxylin followed by a modification of Mallory's tri-acid stain (Everett, 1943).

In the experiments which follow, progesterone was administered subcutaneously in a sesame oil solution containing 5 mg. per ml. Estradiol benzoate was also given in sesame oil by subcutaneous injection. This solution contained 330 μ g. per ml. In certain instances small crystals of estradiol weighing approximately 100 to 200 μ g. were introduced subcutaneously by means of a 16 gauge hypodermic needle and stylus (Everett, 1947).

RESULTS

Five-day cycle

Advancement of ovulation time by progesterone. In a preliminary report (Everett, 1944) as mentioned above, it was shown that in the 5-day cyclic rat, if progesterone is injected near midday on the third day of diestrus, ovulation time is advanced about 24 hours. This basic

experiment will merely be summarized here (fig. 1, B). The reader is referred to the article cited for additional information. In choosing 5-day cyclic animals for this treatment only those showing distinctly leucocytic smears on the day of injection were employed, to insure against any spontaneous shift to a 4-day cycle. When given as a single injection, timed as indicated above, progesterone causes ovulation to occur approximately 24 hours earlier than it would have occurred without treatment. In ovaries removed about noon on the day following injection of the new corpora lutea appear identical histologically with those which one would find a day later in the nor-

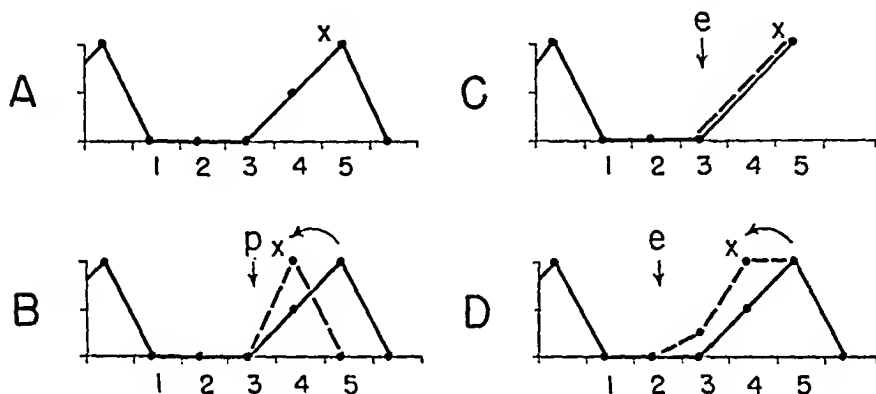


FIG. 1. The 5-day cycle and its experimental modifications. Two units of the ordinate represent a full estrous smear. Time in days is indicated on the abscissa, each unit representing 24 hours (midnight to midnight). Days of the cycle are numbered progressively beginning on the first day of diestrus. Ovulation time is represented by "X".

A. The standard 5-day cycle, ovulation occurring shortly after midnight on day 5.

B. 1.0 to 2.0 mg. of progesterone (p) injected on day 3 accelerates ovulation and vaginal cornification about 24 hours.

C. 33 to 100 μ g. of estradiol benzoate (e) injected on day 3 does not modify the cycle during the next 48 hours.

D. 50 μ g. estradiol benzoate (e) or implantation of small crystals of estradiol on the second day of diestrus accelerates ovulation and vaginal changes 24 hours.

mal 5-day cycle. Dose levels of either 1.0 or 2.0 mg. of progesterone appear to be equally effective in Vanderbilt rats and in hybrids,³ a fact which is especially significant when it is recalled that 1.5 mg. daily will suppress ovulation if treatment is begun sufficiently early in the cycle (Phillips, 1937).

The vaginal smear on the morning after induced ovulation is characteristically fully cornified, a fact indicated in figure 1, B, by the peak in the broken line. Although not of maximal size the cells appear in clumps and present a picture quite commonly seen in the 4-day cyclic rat on the morning after spontaneous ovulation.

³ The failures reported with the larger dose in DA rats (Everett, 1944) may or may not be significant. In the absence of further information in that strain, only the data from Vanderbilt rats and hybrids is considered acceptable at present.

In addition to cases cited in the preliminary report, 5 more positive responses (in Vanderbilt rats) have been obtained. Hence, in 18 of 19 cases (11 Vanderbilt rats, 8 hybrids), ovulation time was advanced about 24 hours and the process of vaginal cornification was significantly accelerated in 17 of the 18 rats. Six controls (2 Vanderbilt rats, 4 hybrids) receiving 0.4 ml. sesame oil in place of the progesterone solution were proestrous on the following day as expected.

Advancement of ovulation by estrogen. There is general agreement that in the rat the full vaginal response to injected estrogen is delayed, so that in primed castrates cornification is not obtained until about 36 to 48 hours after injection (cf. Gustavson, 1939; Jones and Astwood, 1942). With respect to induction of ovulation by estrogen in rats, most information pertains only to immature females where the response is delayed about 4 or 5 days (Hohlweg, 1934; Westman and Jacobsohn, 1938). In the adult female, however, administration of estrogen during early pregnancy is regularly followed by ovulation and new corpus luteum formation within 36–40 hours (Everett, 1947). It is now reported that when estrogen is administered on the second day of diestrus in 5-day cyclic rats, ovulation occurs approximately 24 hours earlier than if no treatment were given (fig. 1, D), again at an interval of approximately 40 hours after injection.

As a preliminary to this particular phase of the study, 4 Vanderbilt rats were injected with estradiol benzoate (33 $\mu\text{g.}$, 3 cases; 100 $\mu\text{g.}$, 1 case) at about noon on the third day of diestrus (fig. 1, C). During the following two days no modification of the normal vaginal smear sequences was found. Tubal ova were observed at autopsy about 48 hours after injection and the newly forming corpora lutea were exactly comparable histologically with those normally found at that stage of the 5-day cycle. Thus no interference with the ovarian cycle was apparent within that time interval and such doses of estrogen appear entirely compatible with the processes involved in follicle maturation and ovulation.

Subsequently, seven 5-day cyclic Vanderbilt rats were given estrogen during the second day of diestrus (fig. 1, D), between 11:00 A.M. and 3:00 P.M. Five of these received 50 $\mu\text{g.}$ of estradiol benzoate in 0.15 ml. of oil. In the other two cases small crystals of estradiol were introduced subcutaneously. On the following morning the vaginal smears were still somewhat leucocytic except for one case in which an early proestrous smear was obtained. On the second morning all smears were cornified. At autopsy later that day, about 48 hours after estrogen treatment, full sets of tubal ova were found in all cases and the ovaries contained full sets of very new corpora lutea which in histological section appeared closely similar to those found a day later in the normal 5-day cycle. Thus, this treatment accelerated vaginal cornification and advanced ovulation about 24 hours.

Four-day cycle

Non-effect of progesterone injected 24 hours before proestrus. Six 4-day cyclic females (hybrids) were injected with progesterone between 10:30 A.M. and 2:00 P.M. on the second day of diestrus, about one day before proestrus was expected (fig. 2, B). Four of them re-

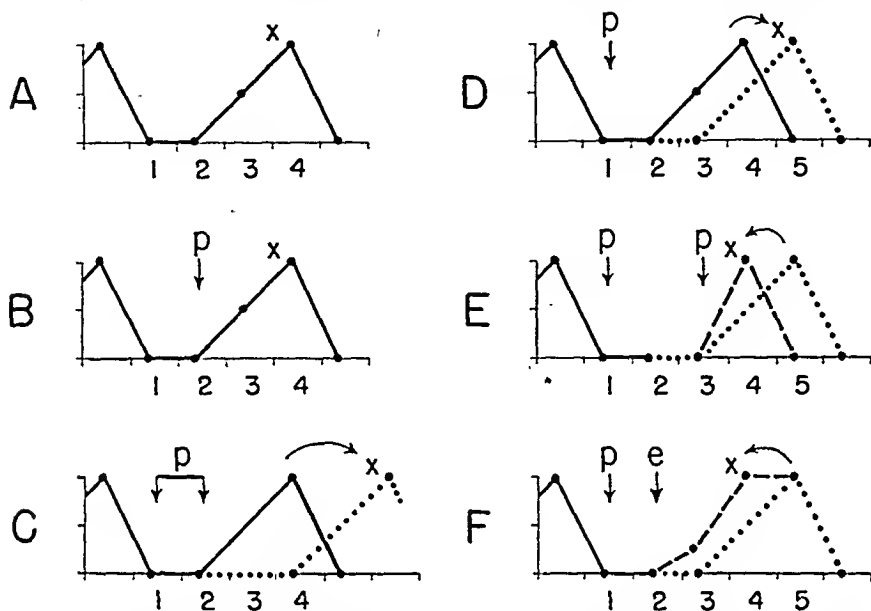


FIG. 2. The 4-day cycle and its experimental modifications. Units on the ordinate and abscissa have the same meaning as in figure 1.

A. The standard 4-day cycle, ovulation occurring 1:00 to 2:30 A.M. early in day 4.

B. Progesterone (p) injected on the second day does not accelerate ovulation and vaginal changes (cf. fig. 1, B).

C. 1.5 mg. of progesterone (p) on days 1 and 2 of diestrus retards the cycle 2 days.

D. 1.5 mg. of progesterone (p) on day 1 of diestrus retards ovulation and vaginal estrus about 24 hours. This is an "artificial" 5-day cycle, now indicated by the numbering below the abscissa.

E. In such an artificial 5-day cycle, additional progesterone on day 3 of diestrus advances ovulation time and vaginal cornification about 24 hours (cf. fig. 1, B and contrast with fig. 2, C).

F. In the artificial 5-day cycle estrogen (e) on the second day of diestrus advances ovulation time 24 hrs. (cf. fig. 1, D).

ceived 2.0 mg. each and two received 1.0 mg. each. On the following morning the smears were of early proestrous type, no evident modification having been produced by the treatment. At autopsy, about 24 hours after injection, the ovaries presented the typical appearance of proestrus, containing sets of large, clear follicles. Histologically they were identical with those in untreated control rats at the same stage of the cycle. The uteri, however, were slender, in contrast with the distended condition normally seen at this time.

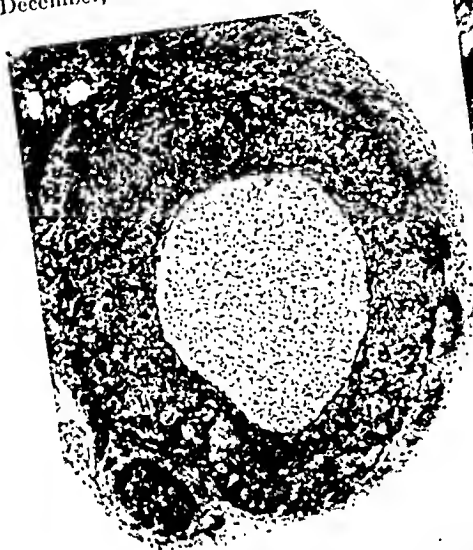
Retardation of the cycle by progesterone injected early in diestrus.

When daily injections of over 1.0 mg. of progesterone are instituted during early diestrus, the diestrus is maintained throughout the period of injection and for 2 days afterward (Phillips, 1937). Then the animal returns to heat. We have repeated this experiment on numerous occasions with the same result. For example, in the particular modification illustrated in figure 2, C, it is seen that, by treating 4-day cyclic females with a limited series of 2 daily injections of progesterone (1.5 mg.) both the onset of vaginal estrous stages and the time of ovulation are retarded about 2 days.

When only one injection of progesterone is given to a 4-day cyclic rat on the first day of diestrus the result is illustrated in figure 2, D: the 4-day cycle is transformed to a 5-day cycle by retarding both the onset of vaginal estrus and ovulation time by about one day. Twenty-nine rats were so treated during a total of 43 cycles. Two different dose-levels were employed. In 23 cycles the amount injected was 1.0 mg. Among 8 such cases in hybrid rats 6 cycles were prolonged one day and 2 were not. In Vanderbilt rats 12 of 15 cycles were prolonged one day. Because of these occasional failures the dose was then increased to 1.5 mg. No failures were encountered among 20 cycles in 18 Vanderbilt rats given this amount. These animals exhibited proestrous smears on the third morning after injection in all cases. Eleven rats were then autopsied without allowing the cycles to continue longer. Their uteri were hyperemic and distended. Their ovaries contained sets of large, clear follicles which were histologically identical with those found during early proestrus in the normal 5-day cycle (fig. 5). They were larger than during proestrus in the 4-day cycle, but gave no evidence of preovulatory swelling. In 10 cases the cycles were continued for at least another day, full cornification appearing at that time. Four were autopsied on that day, on the fourth day post-injection. The reproductive tracts were typical of late estrus. The ampullae of the oviducts were dilated and full sets of tubal ova were demonstrated. New corpora lutea were found, histologically identical with those obtained in normal cycles on the day after ovulation (figs. 3 and 4). In other instances, in which the animals were followed longer, diestrous smears were found on the following morning.

Since these artificially retarded cycles in 4-day cyclic rats are apparently identical with normal 5-day cycles, it appeared desirable to test such cases with respect to the sequelae of injection of additional progesterone on the third day of diestrus or of estrogen on the second day of diestrus. These results are described below.

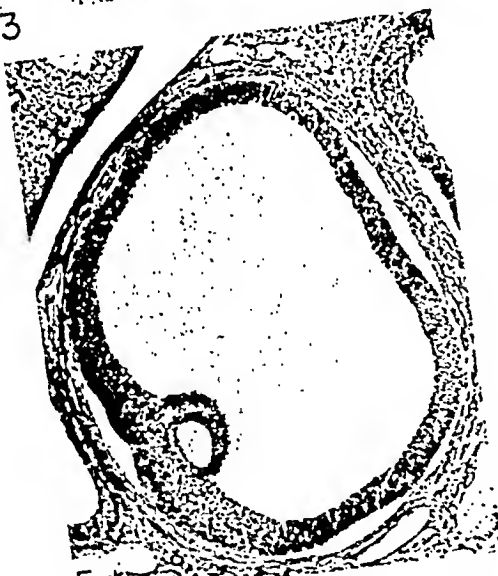
Effect of a second injection of progesterone during diestrus day 3 of the retarded cycle. Five 4-day cyclic Vanderbilt rats were each given 1.5 mg. of progesterone on the first day of diestrus. Two days later, the third day of diestrus in the retarded cycles, each rat received 1.0 mg. of progesterone between 12:00 and 1:00 P.M. (fig. 2, E). On the following morning the vaginal smears were predominantly cornified in



3



4



5



6

FIG. 3. New corpus luteum at 12:00 Noon on day 5 (estrus) of a standard 5-day cycle (cf. Boling, 1942, fig. 3). 80X.
 FIG. 4. New corpus luteum at 12:30 r.m. on day 5 (estrus) of a potential 4-day cycle retarded 1 day by progesterone (cf. fig. 2, D). 80X.

FIG. 5. A follicle typical of day 4 (proestrus) in a 5-day cycle. This specimen was actually obtained during a potential 4-day cycle, retarded 1 day by progesterone injected on day 1 of diestrus (cf. fig. 2, D). Ovaries removed ca. 72 hrs. post-injection. 80X.

FIG. 6. New corpus luteum at 8:30 a.m. on day 4 of an experimental cycle as represented in fig. 2, E. Originally a 4-day cycle; progesterone on day 1 of diestrus and again on day 3 (4:00 r.m.). Closely similar to corpora lutea at this hour of day 4 (estrus) in standard 4-day cycles (6.8 hr. post-ovulatory). 80X.

4 cases, with slight admixture of small nucleated epithelial cells and a few nucleated squamous cells. One smear was recorded as proestrous. The 4 showing cornification were autopsied that morning. Tubal ova and new sets of corpora lutea (fig. 6) were demonstrated in all. The remaining rat was continued until the following morning. At that time the smear had become diestrous and at autopsy the ovaries appeared to contain a set of unruptured follicles and one element tentatively diagnosed as a new corpus luteum. Serial sections of that ovary disclosed 2 large atretic follicles and a corpus luteum, estimated to be about 30 hours old.

Effect of estrogen injected on diestrus day 2 of the retarded cycle. Five 4-day cyclic Vanderbilt rats were injected with 1.5 mg. of progesterone on the first day of diestrus. On the following day (fig. 2, F) each rat received an injection of estradiol benzoate: 50 μ g. in 2 cases, 15 μ g. in 3 cases. They were autopsied about 48 hours later. The vaginal smears registered little significant effect of the estrogen on the first morning after injection, but were fully cornified on the second morning. Examination of the reproductive tracts disclosed hyperemic, distended uteri. The ampullae of the oviducts were dilated and tubal ova were demonstrated in each case. Newly forming corpora lutea were recognized in all 5 pairs of ovaries.

It is thus apparent that in the artificial 5-day cycle, as in the natural 5-day variety, ovulation time can be advanced by either progesterone or estrogen when the injections are suitably timed.

Attempts to ovulate persistent-estrous rats by estrogen therapy

Non-effect of estrogen alone. In a variety of experiments with persistent estrous rats of the DA strain, single injections or multiple daily injections of estradiol benzoate have consistently failed to induce ovulation or luteinization. Only the more significant of these experiments will be described here. Having failed to produce these effects by estrogen therapy while persistent estrus was in progress and having meanwhile devised a means of inducing cycles in such animals by progesterone treatment (Everett, 1940, 1943), the next logical step was to introduce estrogen during diestrus after one completed "progesterone cycle." For purposes of illustration a sequence of two "progesterone cycles" is shown in figure 7, A. Persistent estrus is interrupted by one injection of 1.0 mg. of progesterone after which a diestrous interval of 2 to 3 days usually follows. If, then, a second injection of progesterone is given during proestrus the cycle is completed. Subsequent cycles are completed if progesterone is given during each proestrus. Ovulation occurs in about 70% of the cases.

Twelve persistent-estrous DA rats were each passed through one progesterone cycle as shown in figure 7, B. Six of these also received 1.5 mg. of progesterone (not shown in the figure) on the first day of the second diestrous interval to insure its potential duration for 3

days. All 12 rats received 50 μ g. estradiol benzoate on the second day of the second diestrus as illustrated and were autopsied about 48 hours later. The vaginal smears became proestrous one day after the estrogen treatment and were fully cornified on the second day. At autopsy the only recent corpora lutea present were those representing the progesterone cycle (10 cases). The current sets of follicles gave no evidence of luteinization or preovulatory changes.

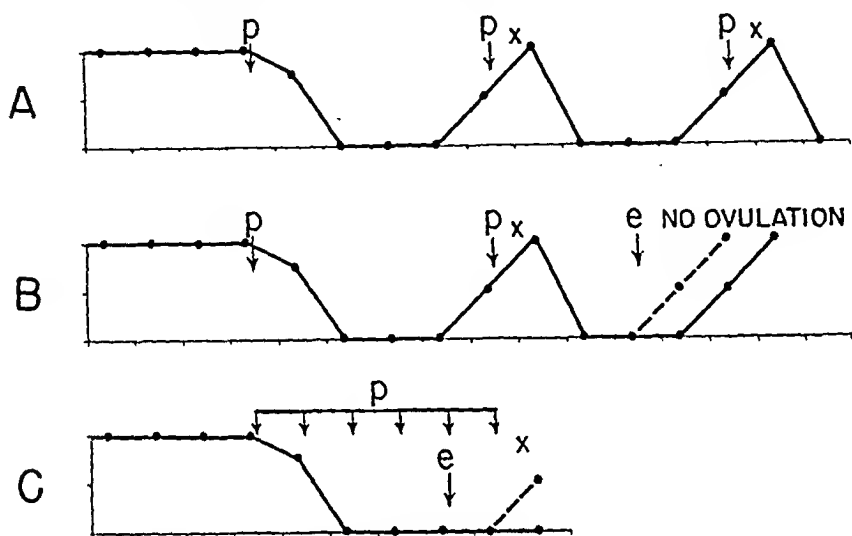


FIG. 7. Experiments with persistent-estrous DA rats. Units of the ordinate and abscissa have the same meaning as in fig. 1.

A. Standard procedure for producing "progesterone cycles," based on data reported previously (Everett, 1943). Each dose of progesterone (p) is 1.0 mg. New corpora lutea induced in about 70% of the cycles.

B. A "progesterone cycle," followed by estrogen (e) on the second day of the second diestrus. No evidence of ovulation 48 hrs. after the estrogen (contrast with figs. 1, D and 2, F).

C. A "pseudopregnancy" maintained by daily injection of progesterone (1.5 mg.). Estrogen induces ovulation in many such cases (see text).

In a correlated experiment, not illustrated, attempts were made to accelerate ovulation time in nine 5-day cyclic, young DA females by administering estrogen during diestrus. The average age was 112 days (range 87 to 147), well before the expected onset of persistent estrus, with two exceptions. Seven of the 9 rats received 50 μ g. estradiol benzoate, 1 received 25 μ g. and 1 received an estradiol crystal implanted subcutaneously. In every instance the estrogen was administered on the second day of diestrus and autopsy was performed 2 days later. In only 2 cases out of the 9 were tubal ova demonstrated and new corpora lutea found. The others had not ovulated and their ovaries contained sets of large follicles. This low score is highly significant when compared with the uniformly positive response obtained in the 5-day cyclic Vanderbilt rat.

Induction of ovulation by estrogen during progesterone-induced pseudopregnancy. From the several studies mentioned above and from our earlier work, it was apparent that progesterone *per se* is not incompatible with ovulation. When a rat receives 1.5 mg. of progesterone or more daily, beginning in early diestrus, a possible reason that ovulation is suppressed is that gonadotrophin (LH?) secretion is diminished to the point where little estrogen is produced or at least no sharp increments of estrogen occur. During pregnancy or pseudopregnancy induced in rats by sterile mating, it is known (Everett, 1947) that administration of estrogen causes ovulation and new corpus luteum formation within 48 hours. Although in DA rats the LH-release mechanism is so apparently refractory to estrogen, especially after reaching the state of persistent estrus, the majority of them respond, nevertheless, by forming new corpora lutea if progesterone is given when estrogen levels are rising (fig. 7, A). It seemed probable that if pseudopregnancies were established in persistent-estrous DA rats by daily injection of progesterone, ovulation and luteinization could then be induced by injection of estrogen. This experiment was performed on 10 rats, as illustrated in figure 7, C. Each animal received 1.5 mg. of progesterone daily for 6 days. On the fifth day each rat also received 50 μ g. of estradiol benzoate. Autopsy was performed on the second day after the estrogen therapy. Ovulation had occurred in 4 of the rats, tubal ova and new corpora lutea being demonstrated. The ovaries of 3 others contained numerous luteinizing follicles which had failed to rupture. The remaining 3 rats had not ovulated, their ovaries containing sets of follicles which were not hyperemic. Histologically, these latter follicles gave no evidence of preovulatory swelling. Thus, positive responses may be claimed for 7 of the 10 rats and even the 4 cases of proven ovulation are highly significant in view of the extreme refractoriness of such rats to estrogen under other circumstances.

DISCUSSION

In light of the above evidence the theory that progesterone action on the hypophysis is merely to suppress LH secretion (and hence to suppress ovulation) appears no longer tenable. Under certain conditions progesterone clearly facilitates the release of luteinizing hormone. Examination of the conditions under which the respective actions occur may assist in resolving the seeming paradox.

Suppression of estrous changes, with postponement of ovulation, occurs when progesterone acts early in the cycle, beginning no later than 2 days before the next expected proestrus (Phillips, 1937; and the above data). At such times estrogen secretion is probably slight. Furthermore, the hypophysis has recently lost much of its LH. There is at present no clear evidence to show whether the LH content remains low during continued action of progesterone. Evans and Simp-

son (1929) found a slight increase of gonadotrophic potency in hypophyses of pregnant rats when compared with glands from the non-pregnant females. Siegert (1933), however, reported a lower potency during pregnancy than during diestrus. The fact that after a regime of progesterone injections (as in fig. 2, C) a measured interval of about 3 days is required for the spontaneous reappearance of proestrous changes, suggests that during the period of action of progesterone the actual LH content of the gland continues at low level. This does not deny continued synthesis of LH. Perhaps under these circumstances it is released as rapidly as it forms and thus never accumulates in quantity sufficient to precipitate ovulation.

A close study of the experiments represented in figures 2, C, and 2, E, will make evident the wide difference of response which results from a relatively small difference in timing of the second injection of progesterone. If after the first injection the second one follows in about 24 hours, the impending estrus and ovulation are retarded an additional 24 hours. If, however, after the first injection one waits 48 hours to give the second one, the impending estrus and ovulation are accelerated. In the former instance the conditions are those discussed in the preceding paragraph. In the latter, by allowing the extra day to intervene, these conditions have changed. We may legitimately believe that on the third day of diestrus estrogen secretion is now considerably elevated and an increased store of LH appears likely. Under such circumstances, either in the retarded 4-day cycle (fig. 2, E) or in the natural 5-day cycle, progesterone facilitates ovulation. In the normal 4-day cycle, however, when progesterone is given 1 day before expected proestrus, no facilitation is obtained (fig. 2, B). In some manner this failure is a function of the shorter interval after the last estrus and the last ovulation. It is reasonable to think that here estrogen secretion is still rather slight (at the time of progesterone injection) and also that elaboration of a store of hypophyseal LH has not yet progressed in adequate degree.

One may suggest that in the phenomena in which progesterone facilitates ovulation estrogen is the primary underlying factor, progesterone acting as a modifier. Hohlweg and Dohrn (1931) reported that while a certain minute quantity of estrogen (1 R.U. of Progynon) is inadequate by itself for prevention of castration-cell formation in the rat hypophysis, the same amount combined with 1 RBT. U. of progestin is entirely sufficient. It is also known that progesterone exerts a "sparing action" upon estrogen (Smith and Smith, 1946), preventing destruction of estrone by the liver. Such action may explain the Hohlweg and Dohrn observation. It may also account for the facilitation of ovulation and acceleration of vaginal cornification by progesterone. There are certain considerations, however, which make it difficult to accept such an explanation outright.

In the first place, it is not yet certain whether estrogens themselves

are responsible for the various actions upon the hypophysis such as LH release, or whether break-down products, such as the lactone suggested by the Smiths, are truly the active agents. If it is such "inactivation" products which are effective, then by preventing inactivation progesterone ought to prevent rather than foster stimulation of the hypophysis. It should be noted that Bradbury (1947) reports failure of two samples of Westerfeld's lactone to produce effects upon the immature rat hypophysis like those induced by active estrogens.

In the second place, it is probable that the stimulus to release of the ovulatory surge of LH occurs within a very few hours after injection of progesterone. Ovulation time is estimated as roughly 15 hours after injection. Judging from data on the rabbit (Fee and Parkes, 1928; Waterman, 1943, and refs. cited there) luteinizing hormone must usually be released in that species 9 to 10 hours before ovulation occurs. In the cyclic rat, it appears that neurohumoral stimulation essential for LH release passes to the hypophysis between 9 and 11 hours before ovulation time (Everett, Sawyer and Markee, 1948). If a similar interval between LH release and ovulation time occurs in the rat after progesterone injection, then the stimulation of the hypophysis must be accomplished within some 5 or 6 hours after administration of the hormone. Thus, if progesterone acts here by preventing estrogen inactivation by the liver, we must imagine the latter steroid(s) to be accumulating at very rapid rate, until a sufficiently high blood estrogen level is attained to stimulate the LH-release mechanism. This or an analogous indirect process may actually occur, but it seems equally plausible that progesterone modifies estrogenic stimulation of the hypophysis more directly, perhaps by lowering the threshold of the LH-release mechanism to estrogen. The experiment with persistent-estrous rats cited above (fig. 7, C) should be recalled, in which it was shown that progesterone therapy for several days renders many of these animals no longer refractory to estrogen.

The assumption in the above paragraphs that progesterone acts through the hypophysis, rather than upon the ovary, is well founded. The evidence in brief is as follows: (1) Induction of ovulation by progesterone in persistent-estrous rats is accompanied by "repair" of the interstitial tissue (Everett, 1940, 1941, 1943). (2) Although not cited in the present report, induction of 'premature' ovulation in 5-day cyclic rats is accompanied by depletion of cholesterol in the interstitial tissue, an indication of LH action (cf. Claesson and Hillarp, 1947), and by increased cholesterol storage in the corpora lutea of the preceding set, a further function of increased LH secretion (Everett, 1947). (3) Induction of 'premature' ovulation in the 5-day cyclic rat by progesterone may be blocked by administration of either dibenamine or atropine in large doses (Everett and Sawyer, unpublished; cf. Sawyer, Everett and Markee, 1948; Everett, Sawyer and Markee, 1948). Since the studies with these drugs in the rabbit

(Sawyer, Markee and Hollinshead, 1947; Sawyer, Markee and Townsend, 1948) clearly imply that they prevent ovulation by blocking neurohumoral stimulation of the anterior lobe (Markee, Sawyer and Hollinshead, 1948), it is most logical to conclude that in the rat progesterone under proper conditions somehow enhances such stimulation.

The acceleration of vaginal cornification by progesterone is a puzzling question. Jones and Astwood (1942), in a detailed study of the responses of primed castrate rats to estrogen and to various combinations of estrogen and progesterone, reported that progesterone causes neither acceleration nor retardation of the vaginal estrous changes during the first 3 to 4 days of treatment. Furthermore, they reported and it is generally recognized (cf. Gustavson, 1939), that a delay of 36-48 hours occurs between administration of estrogen and the onset of distinct vaginal estrous changes. The observed response to progesterone in the 5-day cyclic rat is a far more rapid phenomenon. Furthermore, it is prevented by Dibenamine or atropine treatment (Everett and Sawyer, unpublished) and, hence, is probably caused indirectly by an hypophyseal secretion (LH?). We must, then, conceive of a substance which is secreted by the ovaries(?) some hours after progesterone injection and which acts on the vagina much more rapidly than the estrogens with which we are familiar.

The ability of progesterone to foster ovulation appears at the present writing to result from an enhancement of the action of estrogen. Bradbury's (1947) observation, that in immature rats treated with estrogen the concomitant action of progesterone prevents discharge of gonadotrophin, may appear to controvert such an argument. The conditions in his experiments were, however, distinctly different than those reported herewith. His conclusions cannot legitimately be construed to mean that progesterone always prevents release of gonadotrophin. Neither can the evidence obtained in the rabbit be so construed, for in those instances progesterone was administered for several days preceding copulation (Makepeace, Weinstein and Friedman, 1937) and 24 to 72 hours before administration of copper acetate (Friedman, 1941). Against this evidence should be considered more recent information reported by Klein and Mayer (1946; Klein, 1947). It is known that rabbits will copulate while pregnant, but that ovulation does not then occur (Hammond and Marshall, 1925). However, Klein and Mayer found that when pseudopregnant or pregnant rabbits were treated with estrogen and then mated, new ovulation occurred and a second set of corpora lutea was formed. This is a very significant observation if it can be confirmed—for two reasons. It indicates that estrogen favorably affects release of LH in a species in which such effects have been repeatedly denied. Of more importance for our present argument, it suggests that progesterone suppresses ovulation in the rabbit not directly, but by indirectly lowering

estrogen secretion. When extrinsic estrogen is supplied, progesterone is compatible with release of the luteinizing hormone.

The ideas expressed in this discussion require most cautious evaluation before final conclusions are reached. There can, however, be no doubt that progesterone acts upon the hypophysis in two seemingly different ways depending on other conditions. Whether, in species other than the rat, progesterone actually facilitates release of LH remains an open question.

SUMMARY

In normal rats with predictably regular 4-day and 5-day cycles, respectively, a series of experiments is described, whereby ovulation time and other features of the estrous cycle may be either accelerated or retarded. In the normal 5-day cycle, injection of 1 to 2 mg. of progesterone on the third day of diestrus accelerates ovulation and vaginal cornification about 24 hours. Equivalent results follow administration of certain estrogens on the second day of diestrus. Progesterone does not accelerate the 4-day cycle when injected on the final (second) day of diestrus.

The 4-day cycle is retarded 1 day by injection of 1.5 mg. of progesterone on the first day of diestrus, becoming thus a 5-day cycle. (Continued daily injection of this dose for n days retards ovulation n days.) In such an artificial 5-day cycle, injection of progesterone on the third day of diestrus, omitting treatment on the second day, accelerates the expected ovulation and vaginal changes about 24 hours, just as it does in the normal 5-day cycle. Similarly, in the artificial 5-day cycle, treatment with estrogen on the second day of diestrus accelerates ovulation.

Persistent-estrous rats of the defective DA strain are refractory to estrogen in the respect that ovulation and luteinization cannot be induced in them by estrogen, even when treated during the diestrous intervals resulting from earlier progesterone treatment. However, *during* pseudopregnancies induced in such animals by daily injection of progesterone (1.5 mg.), injection of estrogen has produced ovulation in a significant number of cases.

Thus progesterone "suppresses" ovulation and other features of estrus only under certain conditions. When estrogen levels are elevated progesterone facilitates ovulation, at least in the rat. This it may accomplish by modification of the threshold of the LH-release mechanism to estrogen or perhaps by modification of estrogen metabolism.

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BIO-ASSAY OF CALORIGENIC SUBSTANCES USING TADPOLES OF *XENOPUS LAEVIS*¹

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THE ASSAY of thyroidal and other calorigenic preparations in amphibia has not been completely satisfactory. Recently Deanesly and Parkes (1945 a, b) have devised a new method for thyroidal assay utilizing the eruption of the fore limbs in tadpoles of *Xenopus laevis*. The many obvious advantages of their method may establish it as the most useful procedure for work in this field. However, the time required for the assay (seven days) and the relative insensitivity of the test are disadvantages for certain special problems involving the thyroid. By using the appearance of the fore limb buds as an end-point we have been able to modify the procedure so that the time required has been shortened from seven to three days and the sensitivity has been increased about twentyfold.

MATERIALS AND METHODS

The details of the care, handling and feeding of the adult toads were essentially those described by Deanesly and Parkes. Mating of the adults was secured by injecting 250 and 500 I. U. of chorionic gonadotrophin into the male and female *Xenopus* respectively. A number of commercial preparations, such as korotrin (Winthrop Chemical Co., Inc.) and antuitrin S (Parke, Davis & Company), were used with equal satisfaction. Five batches of tadpoles, varying in number from several hundred to several thousand per batch, were obtained. A week after hatching, the tadpoles were fed liver powder (Armour Laboratories) at a dose of 1 mg. per tadpole per day. The tadpoles were maintained at room temperature (there being no facilities to maintain constant temperature during growth). Within fourteen to twenty-one days they had grown to an optimal size (16 to 24 mm. length) for use. The animals selected for this length were then distributed among 250 cc. beakers, each containing 5 tadpoles in 200 cc. of tap water.

A variety of calorigenic and noncalorigenic substances were tested (table 1). Insoluble substances were passed through a 100 mesh screen and added to the water as a fine suspension. Those compounds readily soluble in water were added in solution. The beakers were then placed in the incubator at $25^{\circ} \pm 1^{\circ}$ C. A range of five or six dosage levels separated at log dose intervals

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TABLE 1. SUBSTANCES ASSAYED FOR METAMORPHIC ACTIVITY

Materials	Range of dosage, micrograms	Iodine,* per cent	Thyroxine,* per cent
dl-Thyroxine	0.1-3.0	65.4	100
Desiccated thyroid†	10-1,000	1.0	0.4
Protamone‡	10-1,000	6.4	3.3
Iodocasein§	10-1,000	6.2	2.6
Radioactive iodocasein§	10-1,000	5.3	2.0
Iodine	1,000-100,000	100	0
Potassium iodide	1,000-100,000	75.9	0
l-Tyrosine	100-100,000	0	0
Casein	100-100,000	0	0
3-5 l-Diiodotyrosine	1,000-100,000	58.6	0

* Analytic methods to be described elsewhere.

† Strong (Parke, Davis & Company, Detroit, Michigan).

‡ Cerophyll Laboratories, Kansas City, Missouri. Protamone is a commercial iodocasein preparation. The trade name is purposely used to distinguish it from our own preparations of iodocasein.

§ Our own preparations, to be described elsewhere.

of about 0.5, plus a set of 20 animals as controls, was usually employed for each assay. Since 10 to 20 animals were tested at each dosage of any material the final assay represented a determination based on from 50 to 120 tadpoles. Some twenty-two assays on a little more than 2,000 animals were carried out.

After three days, the animals were examined under a hand lens and the percentage of animals showing development of the fore limb buds was determined. The fore limb buds did not appear naturally in normal development until after the first month under the conditions described. Active thyroidal preparations induced a rapid development of the limb buds which could be seen quite easily as grayish white bars extending laterally. After the third day the procedure outlined by Deanesly and Parkes was followed in exact detail in order to compare our modification with the original method, since the latter method at this point consists only in changing the water at the third day and determining the number of animals showing eruption of the fore limb buds at the end of seven days.

For analysis of the dose response relation, the percentages of animals showing a positive response were converted into empirical probits and plotted against the logarithm of the dose. The intercept of the line corresponding to a probit of 5 was designated as the median effective dose (M.E.D.). Although this method of calculation is not necessarily a complete method or the most appropriate method of dose response analysis, it has been utilized so that our results could be directly compared with those of Deanesly and Parkes. The M.E.D.'s, thus obtained, represent the dose of substance per 200 cc. of water containing 5 tadpoles and is not to be construed as representing the effective dose per tadpole.

RESULTS

1. *Preliminary Experiments*.—Several sighting tests were performed with iodocasein according to the method of Deanesly and Parkes. The M.E.D.'s obtained fell between 450 and 550 micrograms, close to the average range of 460 to 660 γ found by the foregoing authors. It was noted however, that many animals on lower dosages did not erupt their fore legs but showed accelerated fore limb development which stopped short of eruption. Moreover, the accelerated fore limb development could be seen as early as one or two days after

TABLE 2. THE COURSE OF THE FORMATION AND ERUPTION OF THE FORE LIMBS AT VARIOUS DOSAGES OF CALORIGENIC SUBSTANCES

Materials tested	Dosage, micrograms	Percentage of animals showing "bars" at daily intervals							Percentage of animals showing eruption at daily intervals			
		1	2	3	4	5	6	7	1-4	5	6	7
Protamone	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	5	5	5	30	30	0	0	0	0
	25	0	20	25	50	60	80	90	0	0	0	0
	50	20	50	85	90	100	100	100	0	0	0	0
	100	20	60	100	100	100	100	100	0	0	10	10
	250	20	75	100	100	100	100	100	0	0	10	25
	500	30	80	100	100	100	100	100	0	20	40	55
Iodocasein	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	10	20	0	0	0	0
	25	0	0	10	10	10	15	25	0	0	0	0
	50	0	15	15	15	40	40	40	0	0	0	0
	100	10	26	52	52	60	60	70	0	0	0	0
	250	10	35	80	94	100	100	100	0	0	5	5
	500	25	65	100	100	100	100	100	0	25	45	53
Desiccated thyroid	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	5	25	25	53	53	0	0	0	0
	50	0	15	70	80	80	85	85	0	0	0	0
	100	0	33	74	100	100	100	100	0	0	0	0
	250	0	25	85	100	100	100	100	0	0	0	0
	500	0	25	89	100	100	100	100	0	10	16	16
	1,000	0	25	100	100	100	100	100	0	10	25	25
	5,000	15	50	100	100	100	100	100	0	100	100	100

administration of the dose. These two observations led to the thought that accelerated fore limb differentiation could be used as an end-point, thus shortening the assay and greatly increasing its sensitivity. It became necessary therefore to investigate the temporal sequence of the fore limb differentiation, to analyze the dose-response curves and to study the specificity of the proposed reaction.

2. *The Course of the Formation and Eruption of the Fore Limb Buds*.—A sample of the results obtained at different dosage levels for iodoproteins at daily intervals after administration is shown in table 2. The percentage of animals showing the appearance of the forelimb buds (hereafter referred to as "bars") and the percentage of animals showing eruption of the fore limb ("erupters") were determined. When the results shown in table 2 were plotted against time, it was more easily seen that the rate of response at all doses for bars was most rapid during the first three days. After the third day the rate of response declined. In other assays, not detailed here, the proportion of positive responses increased but slightly after the third day

and in some experiments asymptotic values were reached. In any case, the third day represented the most appropriate time for reading the end-point, since at this time there was the greatest spread among the effects of various doses and since the rate of response either declined appreciably or became asymptotic after this time. Eruption of the fore limbs did not take place until after the fourth day since they were differentiating up to this time. When the number of animals showing eruption was plotted, the temporal sequence was found to be essentially that previously described. It was also interesting that the time curves of eruption paralleled those observed for bars. Inspection of table 2 will indicate the increased sensitivity conferred by using bars as end-point.

3. *Quantitative Aspects.*—The assays obtained are shown in figure 1, where the percentage of response was transformed into probits and plotted versus the logarithm of the dosage. Since each batch of

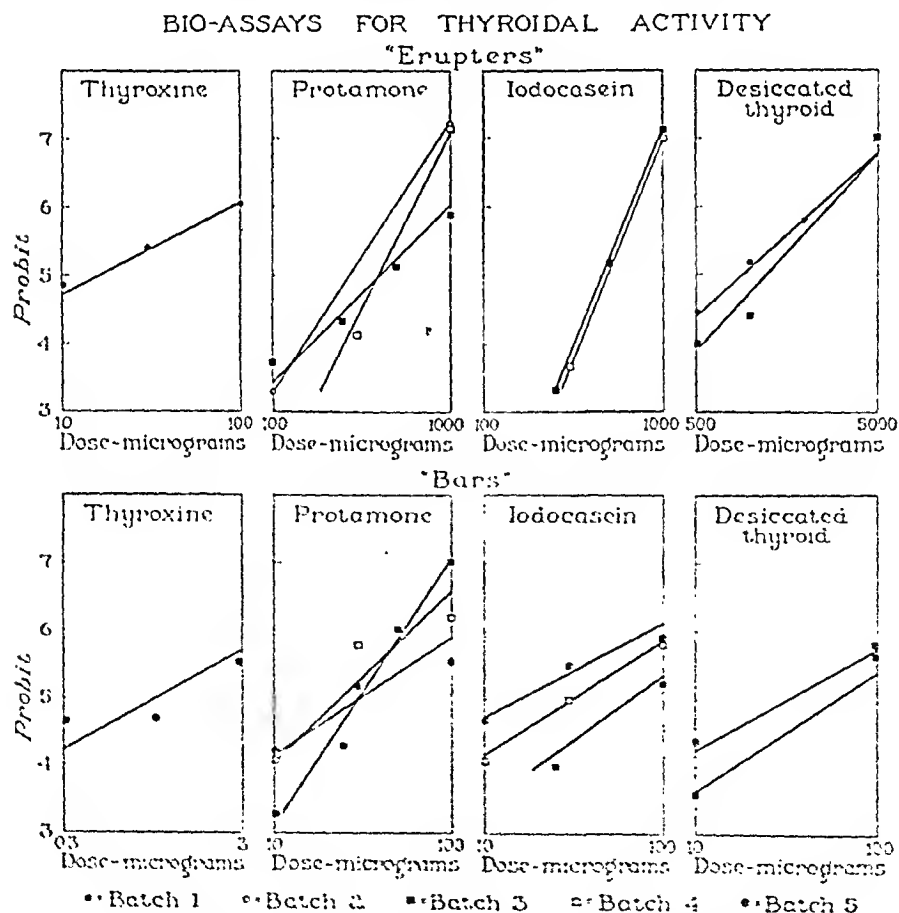


FIG. 1. The dose-response relationship of various calorigenic materials.

tadpoles may show different sensitivity, the individual assays are distinguishable by batch number as well as by material tested. The slopes of the assays using erupters varied from 2.8 to 6.6, a variation that was also encountered in the work of Deanesly and Parkes. Using bars as end-point, the slopes varied from 1.4 to 3.9, apparently somewhat lower values. However, sufficient data are not yet available to indicate whether or not the slopes for bars are generally lower and hence to signify a somewhat lower precision. More satisfactory slopes (that is, greater than 5) presumably can be obtained by further investigation of the dose response curves.

The median effective doses of the various materials are shown in table 3. The results (erupters) compare favorably with those reported for similar substances by the British workers. The ratios of activity

TABLE 3. MEDIAN EFFECTIVE DOSES (MICROGRAMS) OF CALORIGENIC SUBSTANCES

Tadpole batch number	End-point: Fore limb eruption				End-point: Appearance of bars			
	Protomone	Iodo-casein	Thyroid	Thyroxine	Protomone	Iodo-casein	Thyroid	Thyroxine
1			900	16				
2	270							
3	400	480	1,020		30	60	60	
4	400	480			24	30		
5					30	15	32	1
Average	357	480	960	16	28	35	46	1
Ratio (thyroxine =1)	22	30	60	1	28	35	46	1

for most of the preparations (using thyroxine as 1) were roughly of the same order of magnitude when the two end-points were compared. It can be seen from table 3 that an over-all average increase of sensitivity of about twentyfold was achieved. Not shown in table 3 are the results for radioactive iodocasein, tested to assure its biologic potency for use in investigations on the metabolism of iodocasein in human beings. This preparation proved to be one of our best iodocaseins since its M.E.D. was 7γ (bar end-point).

4. *Specificity.*—It has been held by some that the metamorphic changes in amphibia are not specific reactions to thyroidal substances, since materials like iodine and diiodotyrosine also induce such changes. Reineke and Turner (1942), and Deanesly and Parkes (1945 a, b), however, have felt their assays to be highly specific. We are in agreement with the latter opinion for several reasons, the most pointed of which is the available data that miscellaneous substances under the conditions of the test have been found to be inert. Seaweed, potassium iodide, dichlorotyrosine and dibromotyrosine were found by Deanesly and Parkes to be inert at 10 mg. dose levels. Our results show that such additional substances as molecular iodine, tyrosine and casein

(table 1) were inert at high dosage levels when tested by both methods of assay. It should be pointed out that the dose levels shown in table 1 represent maximal tolerated amounts, for the next highest dose for many of the compounds proved lethal. The only "miscellaneous" substance with slight thyroïdal activity was diiodotyrosine. However, the M.E.D. (bars) of this substance was 8.6 mg. or some 6,000 times greater than that of thyroxine run simultaneously. On the basis of the erupter end-point, it was between 600 and 800 times greater than that of thyroxine. Since in routine assay of calorogenic proteins the dosage does not usually exceed 1 mg., it would be quite impossible for any contaminating diiodotyrosine to interfere with the assay or to contribute through its thyroïdal action per se to the result to the extent of more than a few per cent. In the absence of evidence to the contrary, it appears likely that the assay of thyroïdal materials in amphibia is quite specific under the given conditions of the assay.

DISCUSSION

Although the procedure and the results of the modified bio-assay merit little further discussion, the general interpretation of bio-assays for thyroïdal activity using amphibia deserves some comment. Since it is not known precisely to what extent such assays can be applied to mammalian forms, particularly to human beings, the interpretation of results obtained in amphibia may be somewhat hazardous. It is probable that the same calorogenic substances can be utilized quite differently by different organisms and therefore mutual interconversion of values would not be possible. The difference in activity of d-thyroxine and l-thyroxine in various organisms is a case in point. There is, therefore, need in other classes of vertebrates for bio-assay methods similar in convenience and precision to those available for amphibia.

When amphibia alone are used, a number of problems as yet unsolved must be considered. The foremost of these problems is the decision as to which of the many active materials is to be used as the standard of reference for thyroïdal activity. Were the effect of all active substances directly proportional to their thyroxine content, a standard reference of thyroxine=1 could always be employed. Our results, however, would lead us to conclude that the activity of all preparations was not proportional to their thyroxine content. In table 1 the thyroxine content of the various preparations is given. From these data M.E.D.'s (table 3) of the various compounds can be calculated in terms of their thyroxine content. Thus the M.E.D., using the erupter end-point was 16 γ for thyroxine, 12 γ for protamone, 12 γ for iodocasein and 4 γ for desiccated thyroid. Using the bars end-point the M.E.D. was 1 γ for thyroxine, 0.9 γ for protamone, 0.9 γ for iodocasein and 0.18 γ for desiccated thyroid. While the activity of the two preparations of iodocasein would thus appear to be roughly proportional

to their thyroxine content, the activity of desiccated thyroid was four or more times greater than its thyroxine content would allow.

Data of similar nature in terms of acid-insoluble iodine of a great variety of iodinated proteins (ardein, plasma, casein) prepared by Rivers and Randall (1945) have been reported (Deanesly and Parkes, 1945 b). Whether or not acid-insoluble iodine represents thyroxine iodine is problematical. On the assumption that it does, however, the activity of some of the better iodocasein preparations would be only twice as much as their thyroxine content would indicate. Desiccated thyroid was estimated to be some ten to twenty times more potent than iodocasein, since iodocasein containing 1 to 2 per cent of acid-insoluble iodine gave the same physiologic effect as desiccated thyroid containing 0.1 per cent of acid-insoluble iodine. Our results using a more direct determination of thyroxine are in fairly good agreement with the foregoing. The greater activity of desiccated thyroid over artificial iodoproteins in terms of thyroxine or acid-insoluble iodine appears to hold true not only for amphibia but also for birds and mammals.

A different relationship had been observed previously by Reineke and Turner, who found that the activity of iodocasein in *Rana* tadpoles was equal to that of thyroxine in terms of total organic iodine. Two objections to the significance of this relationship have been raised by Deanesly and Parkes: (1) that thyroxine when combined in protein (meaning, presumably, desiccated thyroid) is appreciably more effective than the same amount of pure thyroxine; (2) that a substantial proportion of organic iodine in iodoproteins is acid-soluble iodine, which is largely inert biologically. Calculation of our results in terms of organic iodine (the percentages of iodine for organic compounds in table 1 may be assumed to represent almost wholly organic iodine) reveals a good agreement between desiccated thyroid and thyroxine, but iodocasein would be about a third as active as thyroxine.

Finally, it is of interest to compare the results of the bio-assays with respect to total iodine content, since recently several aspects of the effects of elementary iodine on processes related to the thyroid have received some attention (Albert and co-workers, 1946; Dvoskin, 1947). Many years ago, Swingle (1919) reported that iodine crystals promote metamorphosis in amphibia, and recent work (Dvoskin, 1947; Nelson and Wheeler, 1948) has proved that elementary iodine produces thyroidal effects in mammals. A plausible explanation for this effect is that elementary iodine, when placed directly on or in tissues, induces a local iodination of tissue proteins, some of which are calorigenically active on absorption. Our results (table 1) do not show any relation between activity and total iodine. Furthermore, elementary iodine had no effect when administered in the manner prescribed, indicating that while iodoproteins may be formed at the surface of the

skin of tadpoles, their absorption was nil or negligible to the extent that no biologic response was obtained.

Failure to account for the physiologic activity of various thyroidal substances on some common basis poses a perplexing problem. It might be held that thyroxine as such is less effectively utilized than the same amount of thyroxine in combination with protein. This appears to be true for desiccated thyroid but it is not true, at least not to the same extent, for artificial iodoproteins. The existence of antagonistic substances in artificial iodoproteins which would account for their lesser activity or the presence of facilitating substances in natural thyroprotein which would account for its greater activity when equilibrated with respect of thyroxine content are possibilities that deserve experimental effort. Also, the chemical determination of thyroxine in proteins, the suspicion that acid-insoluble iodine may represent a great deal more than thyroxine iodine and increased precision in bio-assays merit further work.

In view of the foregoing, the present situation is such that there can be no universal standard of reference for activity. Because of the exceptional stability of thyroidal preparations, various compounds can be employed as standards, depending on the nature of the work involved. A reasonable suggestion is that substances having certain general features in common with thyroxine, such as chemical or molecular configuration, size and properties, be referred to thyroxine as a standard. Similarly, iodinated proteins can be used as their own standard. Thus a preparation of iodocasein could be distributed as a standard reference for iodocaseins and the same could be true for iodinated plasma or any other iodinated protein. Natural thyroproteins could be handled in the same manner in addition to the requirements already in force as stated in the United States Pharmacopoeia.

With the availability of convenient and reasonably precise bio-assay methods such as those described for *Xenopus* tadpoles, work of both practical and theoretical import should be accelerated. For routine assays of known, highly active materials, the method of Deanesly and Parkes is very satisfactory. When it is desired to assay small amounts, or especially when only small amounts of active compounds may be suspected, the supplementary method described in this paper for detection and assay may be used to advantage.

SUMMARY

A supplementary method for the bio-assay of thyroidal activity using the appearance of the fore limb buds of tadpoles (*Xenopus laevis*) is described. The significant attributes of this method are its speed and its sensitivity; it thus serves as a useful tool for the detection and assay of small amounts of calorigenic substances.

Various calorigenic substances did not show activity proportional

to their thyroxine content; natural thyroprotein was much more active than its thyroxine content would allow, whereas artificial iodoproteins were roughly proportional to their thyroxine content. Various standards of reference of activity were suggested for the assay of different calorogenic substances.

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FURTHER STUDIES ON THE PROTECTIVE POWER OF ADRENAL EXTRACT AND STEROIDS AGAINST TOXIC AGENTS

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IT HAS BEEN shown that the protective power of adrenal extracts and steroids against the action of a toxic agent, typhoid vaccine, in adrenalectomized rats, can be accurately measured (Lewis and Page 1946). This investigation has now been extended to determine the protective power of adrenal hormone against diphtheria, tetanus and botulinus toxins. Studies were also made to ascertain whether compound A acetate (11-dehydrocorticosterone acetate) and desoxycorticosterone acetate acting together gave greater protection against typhoid vaccine than either one alone. It also seemed of interest to determine the temporal relationship between presence of hormone and protective power against typhoid vaccine.

Because the question must inevitably arise as to whether results with vaccines or toxins in adrenalectomized animals are also related to active infections in normal animals, a study was undertaken of the protective power of adrenal extract in normal mice infected with pneumococci type I and type III.

METHODS

Male Sprague-Dawley rats were adrenalectomized and maintained on 0.9 per cent sodium chloride for drinking (Lewis and Page 1946). The minimal lethal dose (M.L.D.) of botulinus,¹ diphtheria,² and crystalline tetanus toxin² was determined on rats after they had been adrenalectomized for at least 5 days. For comparison, the M.L.D. was also determined on normal rats of like size and age. It was taken as the smallest amount killing all rats within 10 days after subcutaneous injection of botulinus toxin, 96 hours after tetanus toxin, and 72 hours after diphtheria toxin. A concentrated streptococcal toxin was also tested for ability to kill.

The protection afforded by different adrenal hormones against these toxins was studied by injecting adrenalectomized rats with the test solution (if in oil) 48 and 24 hours before 1.0 to 1.5 M.L.D. of toxin and daily thereafter. If the extract was an alcoholic-aqueous solution, injections were made twice daily (48, 40, 24 and 16 hours before the toxic agent and 0, 8, 24, 32

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¹ The botulinus toxin was prepared by Dr. W. A. Starin, Ohio State University.

² The concentrated diphtheria toxin and concentrated streptococcal toxin were supplied by Dr. H. D. Pierma, Lederle Laboratories, and the crystalline tetanus toxin was prepared by Dr. L. Pillemer, Western Reserve University.

... hours after). In testing for protective power, the period of observation after toxin injection was the same as that used in determining the M.L.D.

Rats were treated with compound A acetate and desoxycorticosterone acetate to determine whether synergism occurred in the protective effect against typhoid vaccine. The testing procedure we formerly employed (Lewis and Page 1946) was used and the amount of hormone in oil given per day was: 0.2 mg. compound A acetate, 0.5 mg. desoxycorticosterone acetate, 0.2 mg. compound A acetate and 0.5 mg. desoxycorticosterone acetate, 0.1 mg. compound A acetate, 0.1 mg. compound A acetate and 0.5 mg. desoxycorticosterone acetate.

The temporal relationship between presence of hormone and protective power was studied by giving adrenal extract only at the time of, or after injection of 1.33 M.L.D. typhoid vaccine. One or two toxic protection units (Lewis and Page, 1946) of adrenal extract (aqueous-alcohol) were injected 0, 30 or 60 minutes and 8 hours after vaccine. The number of rats surviving 24 hours after vaccine administration was noted.

The relative protective power of desoxycorticosterone acetate in oil solution given subcutaneously, and in aqueous solution given intravenously or intraperitoneally, one hour before injection of typhoid vaccine, was determined.

Normal mice were treated with adrenal extract and a lethal amount of typhoid vaccine given, to ascertain whether protection was secured. It was found desirable to give two injections of 1.0 ml. typhoid vaccine 16 hours apart to secure uniform lethal effect. Twenty of the mice received one half ml. extract at the time of the two vaccine injections and 8 hours after the second, and 20 received one half ml. extract 32 and 24 hours before the vaccine injections and at the time of the vaccine. Twenty mice received only typhoid vaccine.

To determine whether adrenal extract afforded protection against pneumococci type I and type III, 46 normal female mice weighing 20 to 22 grams were injected intraperitoneally with type I pneumococci and a similar number with type III. An amount was injected which had been found lethal within 96 hours to all animals. Twenty-three of each group were injected twice daily with 0.2 ml. adrenal extract and 23 with 0.2 ml. saline.

RESULTS

The minimal lethal dose (M.L.D.) of botulinus toxin was 0.2 ml. of a 1:100 solution for adrenalectomized and 0.9 ml. for normal rats (chart 1). After 2.2 M.L.D. (determined on normal rats) of toxin, 100 per cent of the animals in both groups died within 24 hours. Such uniformity in speed of lethal action was not observed, however, after smaller amounts of toxin. After 1.0 M.L.D. (determined on adrenalectomized rats) of toxin, the time of death of the adrenalectomized animals varied greatly. Twenty-five per cent died between the 48th and 72nd hour, while a like number succumbed between the 192nd and 216th hour. After 3.75 M.L.D. (determined on adrenalectomized rats) of toxin, such varied death rate was not observed. The dose-response curve was similar to that obtained on normal rats after 1.0 M.L.D.

(determined on normal rats). One hundred per cent of normal rats died between the 24th and 96th hour after 1.0 M.L.D. toxin.

Both adrenalectomized and normal rats showed a serious reduction in food intake and weight loss after injection of toxin. The partial starvation was probably an important factor in causing death of the adrenalectomized animals several days after toxin injection. Normal animals could withstand this additional stress. Ten to 12 days after receiving toxin, surviving animals resumed normal food consumption and showed rapid weight gain.

RELATIVE TOXICITY OF BOTULINUS TOXIN IN NORMAL AND IN ADRENALECTOMIZED RATS

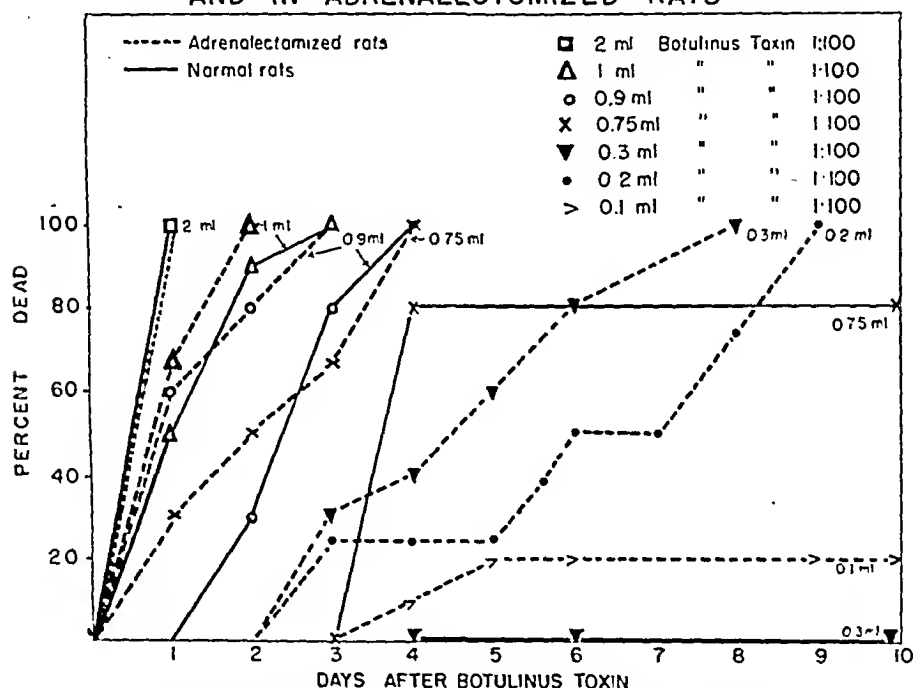


CHART 1. Relative toxicity of botulinus toxin in normal rats and in adrenalectomized rats receiving 0.9 per cent sodium chloride solution for drinking.

Ninety percent of adrenalectomized rats receiving 1.5 ml. adrenal extract were protected against 1.5 M.L.D. (determined on adrenalectomized rats) of botulinus toxin, while only 15 per cent receiving 2.0 mg. desoxycorticosterone acetate survived. The gluconeogenic action of the adrenal extract was probably important in its protective action against the toxin.

As had been found for botulinus toxin, the minimal lethal dose of diphtheria toxin was greater for normal than for adrenalectomized rats. The M.L.D. for adrenalectomized rats was 0.2 ml., and for normal rats 1.0 ml. Adrenal extract (1.5 ml. per day) protected adrenalectomized

tomized rats against as much as 1.5 M.L.D. diphtheria toxin (M.L.D. determined on adrenalectomized rats). Forty per cent of adrenalectomized animals receiving 0.4 mg. compound A acetate per day were protected against 1.5 M.L.D. toxin, while one or two mg. of desoxycorticosterone acetate per day was ineffective.

In contrast with the differences in toxicity of botulinus and diphtheria toxins for normal and adrenalectomized rats, the M.L.D. of crystalline tetanus toxin was 60 mouse M.L.D. for both groups. One mouse M.L.D. is contained in 0.000013 gamma crystalline tetanus toxin nitrogen (Pillemer and Wartman 1947). The speed of action of toxin in normal and adrenalectomized groups was the same and varied

THE RELATIVE TOXICITY OF CRYSTALLINE TETANUS TOXIN IN NORMAL AND IN ADRENALECTOMIZED RATS

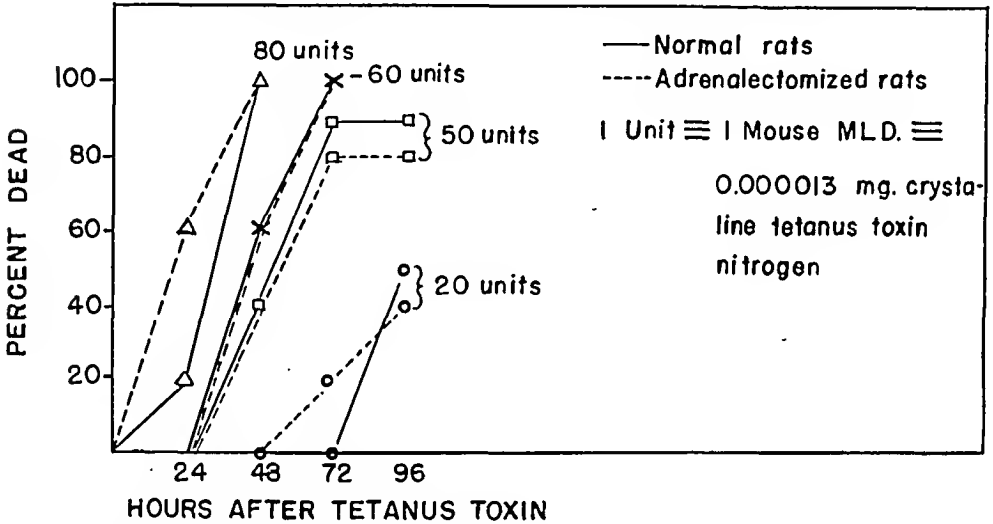


CHART 2. Relative toxicity of crystalline tetanus toxin in normal rats and in adrenalectomized rats receiving 0.9 per cent sodium chloride solution for drinking.

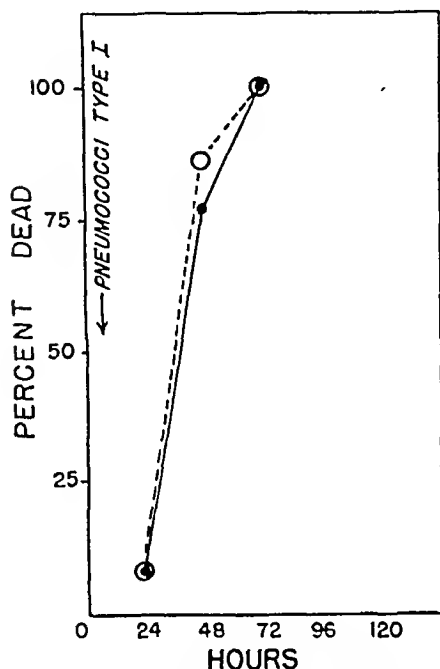
directly with the dose (Chart 2). One and a half ml. of adrenal extract (Upjohn) per day or 2 mg. desoxycorticosterone acetate per day had no protective effective against its lethal action in adrenalectomized rats.

Injection of as much as 2.5 ml. of concentrated streptococcal toxin into adrenalectomized or normal rats produced no evident toxic effects. It appears that both normal and adrenalectomized rats have great natural resistance to this type of toxin.

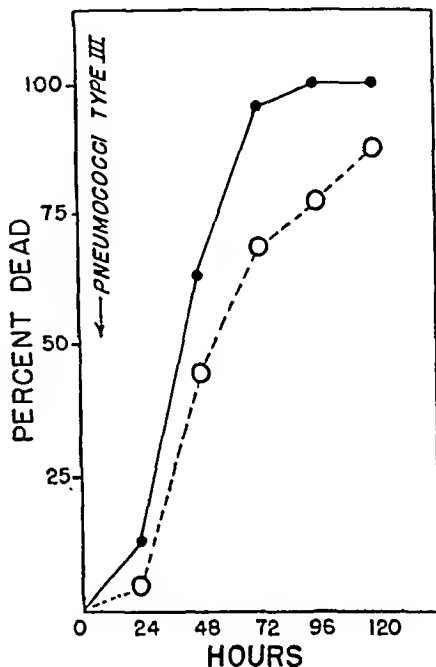
Greater protection of adrenalectomized rats against 1.33 M.L.D. typhoid vaccine was secured when small amounts of compound A acetate and desoxycorticosterone acetate were given at the same time than by either steroid in similar dosage alone. When 0.5 mg. desoxycorticosterone acetate and 0.1 mg. compound A acetate in oil were given 48 and 24 hours before and at the time of administration of typhoid vaccine, 74 per cent of the rats survived. None of those re-

ceiving only 0.1 mg. compound A acetate were alive 24 hours after injection of typhoid vaccine and only 33 per cent receiving 0.5 mg. desoxycorticosterone acetate were protected. All rats receiving 0.2 mg. compound A acetate and 0.5 mg. desoxycorticosterone acetate survived, while 60 per cent receiving 0.2 mg. compound a acetate alone were protected.

GRAPH 1



GRAPH 2



○--- Adrenal extract treated mice
●— Saline treated mice

CHART 3. The relative toxicity of pneumococci type I (graph 1) and pneumococci type III (graph 2) in normal mice receiving injections of 0.2 ml. adrenal extract or 0.2 ml. 0.9 per cent sodium chloride solution, twice daily.

Eighty per cent survival occurred in adrenalectomized rats which received no priming with hormone before the typhoid vaccine, but had received one or two toxic protection units of adrenal extract either at the time of its injection or 30 minutes later. Repetition of the dose 8 hours later, as in the standard procedure, was necessary to attain this survival rate. No greater protection was secured with two toxic protection units per dose than with only one.

Most adrenalectomized animals after typhoid vaccine died within three to four hours. Despite the fact that when hormone is given 30 minutes after its injection there is a high survival rate, when given one hour after, no protection occurs.

Only 10 per cent of rats were protected when 2.0 mg. desoxycorticosterone acetate was given subcutaneously in oil solution 1 hour be-

fore 1.33 M.L.D. typhoid vaccine, while 33 per cent were protected by a similar dose in aqueous solution, given intravenously or intraperitoneally.

Normal mice require two injections of 1 ml. of typhoid vaccine at an interval of 16 hours to kill them. Adrenal extract given at the times of its injection afforded no protection or when given 32 and 24 hours before and at the times of injection of vaccine.

Adrenal extract, when given to mice infected with pneumococci type I, did not modify the fatal course of the disease (Chart 3, graph 1). However, mice infected with pneumococci type III appeared to receive minimal protection, i.e., the speed with which the infection proved fatal was decreased. Further, three of 22 extract-treated mice were alive 120 hours after inoculation, while all non-extract-treated animals were dead in 96 hours, and 96 per cent in 72 hours (Chart 3, graph 2).

DISCUSSION

The protective power of adrenal hormones against the action of bacterial toxin, whether endotoxin such as typhoid, or exotoxin as botulinus, or a combination as diphtheria toxin, seems to parallel its action on carbohydrate rather than electrolyte metabolism. For example, in the protection given adrenalectomized rats against botulinus toxin by adrenal extract, the gluconeogenic action of the hormone probably plays an important part. The food consumption of animals following botulinus toxin is greatly decreased and such partial starvation of adrenalectomized animals results in extreme hypoglycemia. Administration of "carbohydrate active" principles of the adrenal, however, maintains the blood glucose at normal levels. Whether force feeding of adrenalectomized rats during botulinus intoxication would have proved beneficial was not determined.

The synergistic action of compound A acetate and desoxycorticosterone acetate in protecting adrenalectomized rats against typhoid vaccine is interesting. It indicates the desirability of maintaining both electrolyte and carbohydrate metabolism in combatting intoxication.

Evidence indicates that adrenal extract may exert its protective effects rapidly. Death occurred in many rats within 4 hours after typhoid vaccine injection in the absence of extract. But if extract is given as long as 30 minutes after the vaccine, toxic action may be prevented.

That normal animals infected with pneumococci type III were somewhat protected by adrenal extract, while those infected with type I were not, recalls the fact that type III pneumococci are much more toxic. It is possible that the adrenal hormone combatted the toxic rather than invasive component of the infection. Conceivably, in highly toxic infections the use of adrenal extract might be helpful.

Although increased survival of normal rats treated with large doses of adrenal extract subjected to peptone shock has been reported

(Ingle, 1944), extract does not protect normal rats against diphtheria toxin (Ingle, 1947) nor normal mice against typhoid vaccine.

The fact that the lethal dose of crystalline tetanus toxin is the same for normal and adrenalectomized rats is in agreement with earlier observations (Rogoff, 1927) in which less highly purified toxin preparations were used. Grollman (1936) has questioned the completeness of adrenalectomy in the earlier investigations. In the present studies, any possibility of remnants of adrenal tissue was ruled out by testing all surviving rats after the toxin studies for completeness of adrenalectomy and not including results obtained on any animal with evidence of adrenal tissue (Lewis and Page, 1946).

SUMMARY

The M. L. D. of botulinus toxin was approximately 4.5 times greater for normal than adrenalectomized rats. Adrenal extract or compound A acetate was effective in protecting adrenalectomized rats against botulinus toxin.

The M.L.D. of diphtheria toxin was approximately 5 times greater for normal than adrenalectomized rats. Adrenal extract protected adrenalectomized rats, while compound A acetate was only partially effective.

The M.L.D. of crystalline tetanus toxin was 60 mouse M.L.D. for both normal and adrenalectomized rats. No protection was afforded adrenalectomized rats by adrenal extract or desoxycorticosterone acetate.

Adrenal extract (0.5 ml. twice daily) gave no protection to normal mice against lethal doses of typhoid vaccine.

Adrenal extract (0.2 ml. twice daily) gave no protection to normal mice infected with type I pneumococci, while a slight beneficial effect resulted when given mice infected with type III pneumococci.

ACKNOWLEDGMENTS

We are indebted to Dr. M. H. Kuizenga of the Upjohn Company for the adrenal extract used in these studies, to Dr. B. G. Brent of Roche-Organon, Inc., for the special aqueous solution of desoxycorticosterone acetate and some of the oil solution of desoxycorticosterone acetate used, to Dr. Erwin Schwenk of the Schering Corporation for generous amounts of desoxycorticosterone acetate, and to Dr. Randolph Major of Merck and Company for the compound A acetate.

The technical assistance of Mr. William West is gratefully acknowledged.

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GROWTH INHIBITION IN BONE AND BONE MARROW FOLLOWING TREATMENT WITH ADRENOCORTICOTROPIN (ACTH)

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THE ACTION of ACTH on the tibia of immature rats has been studied by Becks, Simpson, Li and Evans (1944) and by Becks, Simpson, Marx, Li and Evans (1944). These workers observed retardation of chondrogenesis and osteogenesis to follow treatment of immature rats with ACTH. Likewise, ACTH antagonized the action of growth hormone when both were injected into hypophysectomized rats. The experiments now being reported supplement these observations by adding information regarding the action of ACTH on the bones of (1) adult rats, (2) under varied dietary conditions controlled by tube feeding and (3) at higher dose levels. In addition, the effect of ACTH on the bone marrow and vertebrae will be discussed.

MATERIALS AND METHODS

The general plan of attack for these experiments has been outlined in detail elsewhere (Baker, Ingle, Li and Evans, 1948). Adult male rats (Sprague-Dawley strain) were force-fed diets high in carbohydrate, fat or protein. Information is given in table 1 concerning the doses of ACTH injected, the duration of the experiments and the number of animals used. The ACTH was prepared by the method of Li, Evans and Simpson (1943) and each daily dose was divided and given in 8 injections. The controls received 0.9% NaCl. At autopsy, the proximal ends of the tibia and 2 to 3 vertebrae were removed for study, decalcified in 5% trichloroacetic acid, sectioned sagittally and stained with hematoxylin and eosin and the Masson procedure. The width of the epiphyseal cartilage was determined microscopically by taking the average of three measurements, one from the center of the cartilage and one each near the anterior and posterior extremities.

OBSERVATIONS

Tibia

The administration of ACTH to adult male rats caused histological modification of the epiphyseal cartilage, the zone of bone formation and of the bone marrow. Since the duration of the experiment proved to be an important factor in the induction of these changes, the effects of treatment over the longest period of time and at the highest dosage (3 mg. per day for 21 days) will be described first.

Epiphyseal cartilage. As is apparent from table 1, measurement of the epiphyseal cartilage showed it to be considerably more marrow in the experimental than in the control rats. This reduction in width resulted from a number of intrinsic changes. First, inhibition of cartilage cell proliferation was indicated by the significant reduction in the number of cartilage cells in the epiphyseal plates. Additional evidence of this suppression was furnished by degenerative changes which were observed in the cells of the proliferation zone (figs. 1 and 2). A majority of these cells were atrophic and possessed pycnotic nuclei. In addition, the cell columns in some cases were somewhat more irregularly arranged. This atrophy of the epiphyseal cartilage was reflected also in the zone of cell and lacunar enlargement. Here, as compared with the controls, there was definite interference with the enlargement of the cells and fewer of them contained intra-cellular vacuoles. Also, the lacunae failed to enlarge as extensively as in the controls. In the treated rats, the invasion of the enlarged lacunae by medullary blood vessels was impaired significantly.

Second, the epiphyseal cartilage was thinner after treatment with ACTH because of reduction in the amount of matrix which it contained. This change appeared to have resulted from the impaired capacity of the atrophic cartilage cells to produce it. In some of the treated rats the matrix, except for the lacunar capsules, stained less intensely with aniline blue suggesting that the chemical or physical character of the matrix had been modified. This was a rather variable observation.

Zone of bone formation. In the control rats, numerous large, active osteoblasts were present in the area of bone formation near the epiphyseal cartilage (fig. 1). In contrast, after treatment with ACTH bone formation was suppressed as shown by a profound and general atrophy of these osteoblasts (fig. 2). It was more difficult to ascertain whether or not the osteoclasts were affected also. They were still present after treatment with ACTH but in some cases may have been reduced slightly in number. They were not affected as noticeably as the osteoblasts.

The impaired osteogenesis manifested itself in great variation in the pattern of the metaphyseal trabeculae. In the controls, most of the inter-columnar septa of cartilaginous matrix projected into the metaphysis as calcified spicules. Frequently, they extended for a short distance without a covering of newly-formed bone. Treatment with ACTH induced variable changes in this osteogenetic region. In some of the rats at the 3 mg. dosage, many of the inter-columnar septa did not project as spicules, leaving considerable areas of the osteogenetic zone free of bone trabeculae. This suggested that in these cases resorption of the labile metaphyseal trabeculae was proceeding more rapidly than their construction. However, in all of the other cases treated at this dose level, the bony trabeculae were broader, "gnarled" in ap-

pearance and less regularly arranged than in the control animals (fig. 2). Likewise, osseous tissue tended to cover completely all of the cartilaginous spicules even extending up on to the epiphyseal plate itself (fig. 2). This was suggestive of the beginning of a "sealing off" of the cartilage by bone which has been observed to occur after hypophysectomy in the rat. The line of junction of the epiphyseal cartilage with the zone of bone formation was quite irregular. This probably resulted from a combination of impaired invasion of the cartilage lacunae and retarded osteogenesis. A significant widening of the marrow sinusoids was not observed in contrast to observations made in immature rat (Becks, Simpson, Li and Evans, 1944).

Detectable modification of the shaft or bony portion of the epiphysis did not occur.

Bone marrow. At the 3 mg. dosage level, ACTH caused a consistent atrophy of the bone marrow which was particularly striking in the center of the shaft (figs. 3 and 4) where normally the red marrow is most dense. This atrophy also involved the marrow of the region of bone formation and, usually the epiphysis as well. The character of this change resembled somewhat "serous atrophy" which has been observed in the bone marrow under various experimental conditions such as under-feeding. In the usual histological preparations, it appeared that fat had increased at the 3 mg. dosage level (3 experimental and 3 control rats) and an attempt was made to verify this observation by staining frozen sections of the bone marrow for fat. The observations made on these preparations of the relative amounts of fat in the marrow of the control and experimental rats were inconclusive. However, it should be pointed out that we did not take precautions to select sections from comparable regions of the bone marrow. These preparations for fat did show that the large spaces of figures 3 and 4 contained fat and, therefore, it seems safe to infer that fat was increased in the bone marrow of the tibia after injection of ACTH. Many megakaryocytes remained in spite of the extensive atrophy of the marrow and did not seem to have undergone much of a decline in number.

Insofar as could be ascertained by histological study, modification of the diet did not affect consistently the structural changes which followed injections of ACTH.

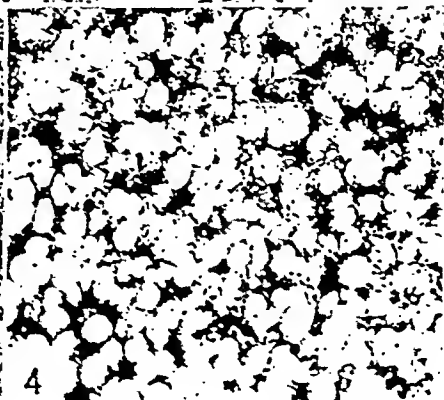
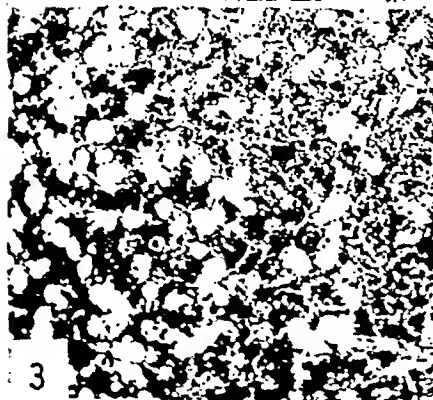
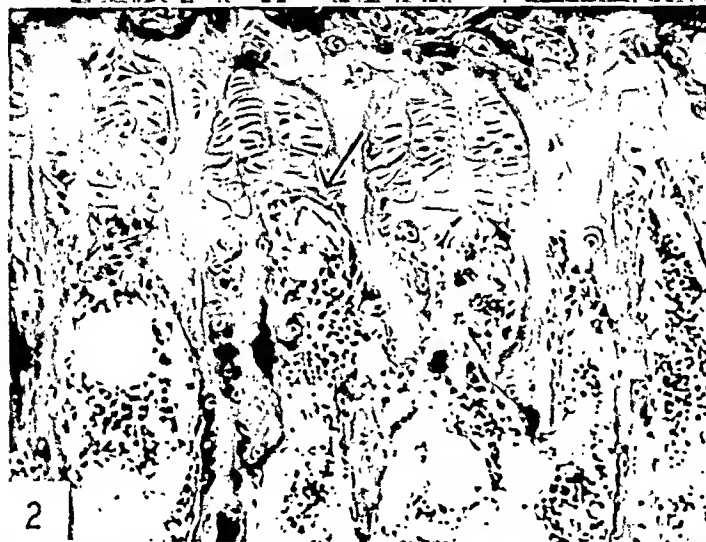
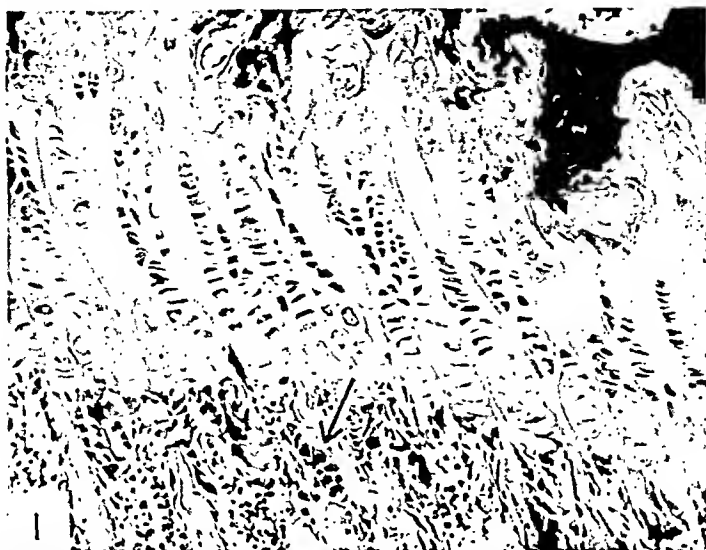
FIG. 1. Rat 901. Control, high carbohydrate diet. Epiphyseal cartilage of tibia. At the end of the pointer are several active osteoblasts. Spicules of cartilage, on which bone has not yet been laid down, extend into the osteogenetic zone. Masson. $\times 184$.

FIG. 2. Rat 1001. One mg. ACTH daily for 21 days, high carbohydrate diet. Cartilage is thinner than in figure 1. There are fewer cells in the columns and those of the proliferating zone are atrophic. The cells of the resorption zone are few in number and smaller. None of the smaller lacunae are being invaded by capillaries. Osteoblasts are atrophic and some bone (arrow) caps the epiphyseal cartilage. Masson. $\times 184$.

FIG. 3. Rat 18003. Control, medium carbohydrate diet.

Bone marrow of shaft. Masson. $\times 75$.

FIG. 4. Rat 17003. Three mg. of ACTH daily for 21 days, medium carbohydrate diet. The red marrow is atrophic and seems to be replaced by fat. Masson. $\times 75$.



On the 1 mg. dosage for 21 days the effects on the bones and bone marrow were essentially similar to those described for the 3 mg. dosage level. The atrophy of the red marrow and the inhibition of chondrogenesis and osteogenesis were equally as marked at this lower dose. Although the one ACTH-treated, protein-fed animal did not exhibit a reduction in width of the epiphyseal cartilage, nevertheless, all of the other histological changes which have been shown to follow such hormone treatment were present.

In general, treatment with 8 mg. of ACTH per day over the shorter period of 10 days did not affect the bone as much as did treatment at

TABLE 1. EFFECT OF VARIED DOSES OF ACTH ON WIDTH OF THE EPIPHYSEAL CARTILAGE

Diet	1 mg. daily for 21 days		3 mg. daily for 21 days		8 mg. daily for 10 days	
	No. Rats	Mean width (μ)	No. Rats	Mean width (μ)	No. Rats	Mean width (μ)
Carbohydrate						
Experimental	2	102	5 ¹	118	4	129.3
Control	2	218	4 ¹	165	2	166
Fat						
Experimental	2	110	2	97	2	139
Control	2	142	1	148	2	147
Protein						
Experimental	1	162	2	97	2	132
Control	1	154	1	148	1	159

¹ Three experimentals and 3 controls were fed a medium instead of high carbohydrate diet.

the lower doses for 21 days. As shown by table 1, it was possible to demonstrate a narrowing of the epiphyseal cartilage by direct measurement. However, significant histological modifications were not readily evident upon microscopic examination. Thus, the effect on chondrogenesis and cartilage resorption was not very great. Impairment of osteogenesis had occurred as shown by a definite reduction in the number of osteoblasts. Likewise, the "gnarling" and distortion of the trabeculae appeared under this dosage, in most cases with bone having been deposited on the cartilaginous spicules up to the epiphyseal plate itself. Atrophy of the red marrow occurred although not as extensively as after the longer period of treatment.

Vertebra

The bodies of the vertebrae were studied in the rats treated for 21 days with a daily dose of 1 or 3 mg. of ACTH. In contrast to the tibia, it was far more difficult to detect histological changes. No consistent modification was found in the cartilage at the superior and inferior ends of the vertebrae, in density of the trabeculae or in thickness of the shaft. The bone marrow showed a slight atrophy of the red marrow after ACTH with some reduction in the density of the constituent

cells usually being evident. However, these effects did not approximate in intensity those which occurred in the tibia.

DISCUSSION

The retardation of chondrogenesis and osteogenesis assumes its greatest significance when viewed in the light of the growth-inhibiting potentiality of ACTH. Apparently, these end-effects are elicited through the stimulated release by the adrenal cortex of the 11-oxy-steroids. The literature supporting the concept of growth inhibition by ACTH and the 11-oxy-steroids is considerable and has been reviewed elsewhere (Baker and Whitaker, in press). It has been shown that numerous proliferating tissues show evidence of atrophy after treatment with ACTH. Among these might be listed the lymphoid tissue (Dougherty and White, 1945), epiphyseal cartilage (Becks Simpson, Li and Evans, 1944), hair and epidermis (Baker, Ingle, Li and Evans, in press).

A negative nitrogen balance was induced in these rats by ACTH (Ingle, Prestrud, Li and Evans, 1947). Presumably, at least part of this loss of nitrogen was the end-result of acceleration of the process of gluconeogenesis in the liver. To what extent tissues other than the liver contribute to this loss of nitrogen is not known at the present time. Also, it is not clear whether this loss of nitrogen is a cause or an effect of the growth inhibition induced by ACTH. The local inhibition of hair and epidermal growth by one of the 11-oxy-steroids (11-dehydro 17-hydroxycorticosterone) shows that the liver is not necessarily a link in the chain of events leading to growth inhibition (Whitaker and Baker, in press).

The bone marrow changes should be considered from 2 view-points: the effect of ACTH on (1) protein metabolism and growth and (2) on fat metabolism. It is probable that the atrophy of the red marrow fits into the pattern of growth inhibition of proliferating tissues brought about by ACTH and, thus, the probable augmentation of fat in the marrow may be secondary to the reduction in volume of the red marrow. On the other hand, a considerable body of evidence shows that the adrenal cortex may be involved more directly in lipid metabolism, especially in its transport (Ingle, 1943). In fact, fatty infiltration of the liver of rats treated with ACTH and fed high carbohydrate diets has been demonstrated previously (Baker, Ingle, Li and Evans, 1948).

The marked atrophy of the red marrow of the tibia after treatment with ACTH suggests the possibility that either an anemia or a granulocytopenia might exist in these animals. Such blood studies have not been reported in the rat but in the mouse White and Dougherty (1945) found ACTH to elicit an increase in the number of red cells and polymorphonuclear leucocytes. These seemingly contradictory findings are difficult to evaluate at this time. It is to be noted that all bone marrow may not share in the atrophy which occurs in the tibia.

At least, the marrow of the vertebrae showed little change.

To a variable degree the changes which we have found to be elicited by treatment of rats with ACTH resemble those which follow under-feeding of rats (Saxton and Silberberg, 1947) and the feeding of diets deficient in various factors of the vitamin B complex to mice (Levy and M. Silberberg, 1946; Levy and R. Silberberg, 1946; Nelson, Sulon, Becks and Evans, 1947; Silberberg and Levy, 1948; Silberberg, Levy and Younger, 1948). This similarity raises the question of the nutritional condition of our animals. Food intake was controlled by tube-feeding. The possibility that ACTH may impair absorption through the intestinal wall has not been examined. Likewise, such similarities of response under these varied experimental conditions suggest an inter-relation between the action of vitamins and ACTH on the peripheral tissues.

The findings in this study are of interest in relationship to the experimental reproduction of the symptoms of Cushing's disease. Especially is this true in view of the position of Albright (1947, p. 329): "... I think it is safe to conclude that whatever bone changes are found in Cushing's Syndrome are the result of an excess production of 'S' hormone." By "S" hormone Albright refers to at least some of the adrenal cortical steroids which we have designated as the 11-oxy-steroids (gluconeogenic) and which, on the basis of physiological grounds (Ingle, Prestrud, Li and Evans, 1947) and the similarity of the effects of ACTH (Baker, Ingle, Li and Evans, submitted for publication) and 11-oxysteroids (Baker and Whitaker, in press) on the skin, appear to be the substances which are secreted at an accelerated rate when the adrenal cortex is stimulated by Li's ACTH.

Many of the symptoms of Cushing's syndrome have been reproduced experimentally by the administration of ACTH or adrenal cortical steroids. Of interest here is the possible appearance of osteoporosis. As pointed out by Albright (1947, p. 296): "In osteoporosis the decrease of bony tissue is due to the fact that the osteoblasts lay down too little bony matrix; that matrix which is laid down is normally calcified." Over the period of our experiment we did not induce osteoporosis. The essential condition for its appearance, however, had been elicited, namely, a suppression of osteoblastic activity. It seems entirely possible that if the suppression of bone formation were maintained by prolongation of treatment with ACTH that the continued resorption of bone to meet the usual bodily demands ultimately might lead to a state of osteoporosis. This outcome would be dependent on at least a normal rate of resorption. The extent to which resorption is affected by ACTH was not ascertained from our studies. The recent finding of Bartter, Forbes and Albright (1948) that treatment of human cases of panhypopituitarism, ovarian agenesis and osteitis deformans with ACTH may cause an increased excretion of urinary calcium is pertinent in this connection. They explained this effect on the basis of continued destruction of bone in the face of failure of os-

teoblasts to form bone matrix when under the influence of adrenocorticotropin.

The concept outlined above holds that the primary effect of ACTH on bone is suppression of bone formation rather than increased withdrawal of bone. In respect to bone, this concept agrees with the hypothesis of Albright (1947) which postulates that the action of the "S" hormones is an "anti-anabolic" one rather than "catabolic." Thus, it appears probable from a study of the effect of ACTH on bone that in the peripheral tissues the 11-oxysteroids inhibit the synthesis of protein rather than accelerate its break-down.

In the vertebrae, where in human Cushing's disease osteoporosis is more likely to occur, we did not find evidence of osteoporosis and only an insignificant difference in osteoblastic activity between the experimentals and controls. These findings do not preclude the possibility that osteoporosis might be reproducible in the rat with ACTH. There is evidence that a refractory state develops during repeated injections of this hormone (Anderson, Page and Li, 1947). Thus, with continued treatment the rats may have been losing their capacity to respond to the action of ACTH.

SUMMARY

The following changes were observed in the tibias of adult male rats treated with ACTH in doses of 1 and 3 mg. daily for 21 days: (1) retardation of chondrogenesis and osteogenesis and (2) atrophy of the red marrow with apparent replacement of it with fat. Variation of the diet which was controlled by tube-feeding did not modify these results significantly. Less noticeable changes were observed in the vertebrae.

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INFLUENCE OF QUANTITATIVE THYROPROTEIN TREATMENT OF HENS ON LENGTH OF INCUBATION PERIOD AND THYROID SIZE OF CHICKS¹

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PREVIOUS WORK in this laboratory has shown that chicks hatched from eggs laid by thyroprotein-fed hens have greatly enlarged thyroids (Wheeler and Hoffman, 1948a). The presence of goiters suggested that the chicks might be hypothyroid and therefore, might exhibit reduced rate of embryonic development. A preliminary study of this aspect of the problem established that eggs of hens fed thyroprotein at a dietary level of 0.02% required 6 to 12 hours longer to hatch (Wheeler and Hoffman, 1948b). Both of these findings have recently been confirmed by the independent studies of McCartney and Shaffner (1948).

The present study was designed to determine the interrelationships between degree of goiterogenicity, increase in length of incubation period, and level of thyroprotein fed with the view of attempting to explain the mechanisms involved.

MATERIALS AND METHODS

To secure eggs for hatching, 60 year-old New Hampshire hens were divided at random into four groups of 15 birds. The hens were kept on litter-covered floors in adjacent pens within the same house. A single male of the same stock was rotated daily among the pens in order to minimize genetic variability of the chicks. One setting of eggs, made during a pre-treatment period established that chick thyroid weight and mean incubation time were comparable among groups. Synthetic thyroprotein² was then added to the diet of three of the groups of hens at levels of 0.02%, 0.04%, and 0.08% respectively, while a fourth group was continued on the control diet. The formula of the basal diet has been published previously (Hoffmann and Wheeler, 1948). Eggs were set for hatching after the hens had been on this regime for an average of 14, 21, and 28 days.³ Treatment was then discon-

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² Synthetic thyroprotein (iodinated casein) was generously supplied by Dr. W. R. Graham, Jr., Cerophyl Laboratories, Kansas City, Mo. This product contains 3.0% thyroxine according to the manufacturer's chemical assay.

³ Eggs were collected during a period of at least seven days before setting. Eggs representing an average of 14 days on treatment were laid from the 10th through the 17th days, $(10+17)/2=14$; 21 days = 18-24; 28 days = 25-31.

tinued and all groups were fed the control diet. Eggs were set again when the hens had been off the experimental diets for an average of 4, 12, 19, and 29 days,⁴ in order to determine the residual effects of treatment.

Length of incubation period was determined by counting the number of chicks appearing in two-hour intervals during the hatch. A total of 866 chicks was involved. Mean incubation time was calculated and evaluated by analysis of variance for subsamples with different numbers of individuals (Snedecor, 1946).

On the day following hatching, a sample of chicks from each group was sacrificed by decapitation, the thyroids were quickly removed and weighed to 0.1 mg. on a Roller-Smith balance. Each sample contained five males and five females.⁵

Since it is well known that seasonal factors influence rate of thyroidal activity it is important to note that these observations were made from February through May 1948.

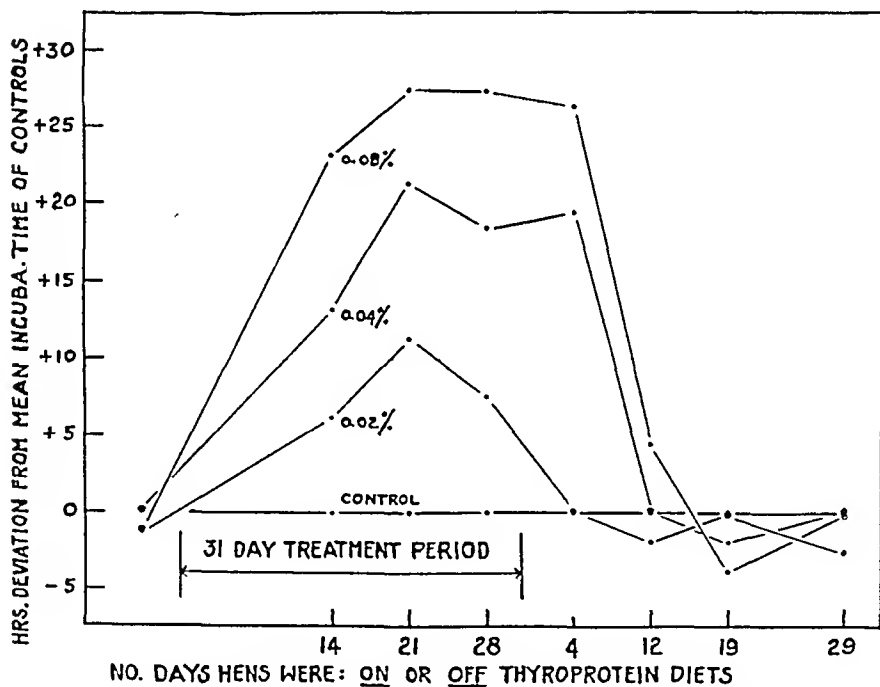


FIG. 1. Delayed Hatching of Chicks Following Administration of Quantitative Levels of Synthetic Thyroprotein to their Dams.

RESULTS

Delayed hatching effect. Length of incubation time was almost proportional to the amount of thyroprotein administered to the hens.

⁴ An average of 4 days = 0-8; 12 days = 9-15; 19 days = 16-22; 28 days = 23-32.

⁵ Day-old female chicks consistently show slightly larger thyroids than male chicks. When small numbers of chicks are compared it is difficult to show statistical significance of this difference. Aberle and Landauer (1935) were able to show a significant sex difference with more than 900 weights. Our unpublished data confirm these findings.

Mean incubation time was increased 6–11, 13–21, and 23–37 hours respectively when thyroprotein was fed at levels of 0.02%, 0.04%, and 0.08% of the diet (Figure 1, and Table 1). The ratio of response was 1:2:3, whereas the ratio of treatment was 1:2:4.

It will be noted (Figure 1) that the maximal effect occurred almost at once and that there was no cumulative increase in incubation time after an average of 14 days on the experimental diets.

Statistical analysis of the data shows that the differences between each level, as well as between treatments and control are highly significant except in three instances in which significance lies at the 5.0% level.

TABLE 1. MEAN TIME REQUIREMENT FOR INCUBATION OF EGGS COLLECTED BEFORE, DURING, AND AFTER THYROPROTEIN TREATMENT OF HENS

Level thyro-protein in diet of hens	Mean length of incubation period (Hrs.) ¹							
	Before treatment	Ave. no. days hens were on exptl. diets			Ave. no. days hens were off experimental diets			
		14	21	28	4	12	19	29
None	519	517	518	519	518	515	518	516
0.02%	518	523	530	526	518	513	518	514
0.04%	519	540	540	537	537	516	517	516
0.08%	518	550	546	546	544	519	515	516

¹ All values for 14, 21, 28 days *on*, and for 4 days *off* the experimental diets are highly significantly different from each other except as noted in text.

The delayed-hatching effect had disappeared within an average of 12 days after treatment was discontinued. There was, however, evidence of residual effect. After an average of 4 days off treatment mean hatching time was normal for the 0.02% lot but the delay persisted at the 0.04% and 0.08% levels. The latter values are highly significantly different from the control value; however, the difference between 0.04% and 0.08% diets is significant only at the 5.0% level.

Other data on the delayed hatching effect involving large numbers of chicks (160 controls and 400 experimentals in two hatches) from hens fed thyroprotein at the 0.02% level, have been previously presented (Wheeler and Hoffman, 1948b). Examination of these data shows the delayed hatch follows a normal curve which is somewhat platykurtic as compared to the curve for the control chicks.

Goiterogenic effect. Although the thyroids of chicks from hens on the experimental diets were, in every case, highly significantly heavier than those of the control chicks, the degree of goiterogenicity was not directly proportional to the amount of thyroprotein in the hen's diet (Table 2, Figure 2).

Differences between the 0.02% level and either the 0.04% and 0.08% levels are highly significant for all hatches of eggs from treated hens as well as for the hatch of eggs collected an average of four days

TABLE 2. MEAN THYROID WEIGHT OF TEN-CHICK SAMPLES (5 MALES, 5 FEMALES) BEFORE, DURING, AND AFTER TREATMENT OF THEIR DAMS WITH THYROPROTEIN

Level thyro-protein in diet of hens	Mean thyroid weight (mgs.) of 2-day-old-chicks							
	Before treatment	Av. no. days hens were on exptl. diets			Av. no. days hens were off experimental diets			
		14	21	28	4	12	19	29
None	4.92	3.87	3.66	3.62	3.43	5.00	5.25	4.89
0.02%	4.90	5.28†	6.30†	5.45†	5.98†	5.25	3.90*	4.35
0.04%	5.14	8.50†	8.68†	8.02†	8.97†	6.55	4.20	4.29
0.08%	5.19	8.35†	7.84†	7.23†	6.99†	4.99	3.75*	4.25

† Highly significantly heavier than corresponding control thyroids (1.0% level).

* Significantly lighter than corresponding control thyroids (5.0% level).

after the control diets were resumed. The difference between the 0.04% and the 0.08% level is statistically insignificant in all cases except one, four days after treatment ceased, when the thyroid weights of chicks from the 0.08% level hens were significantly smaller. Moreover, hens fed the 0.04% level consistently produced chicks with the heaviest thyroids. Next in descending order of thyroid weight were the chicks of hens on 0.08%, 0.02% and control diet (Figure 2). Thus it is apparent the relationship between level of thyroprotein fed

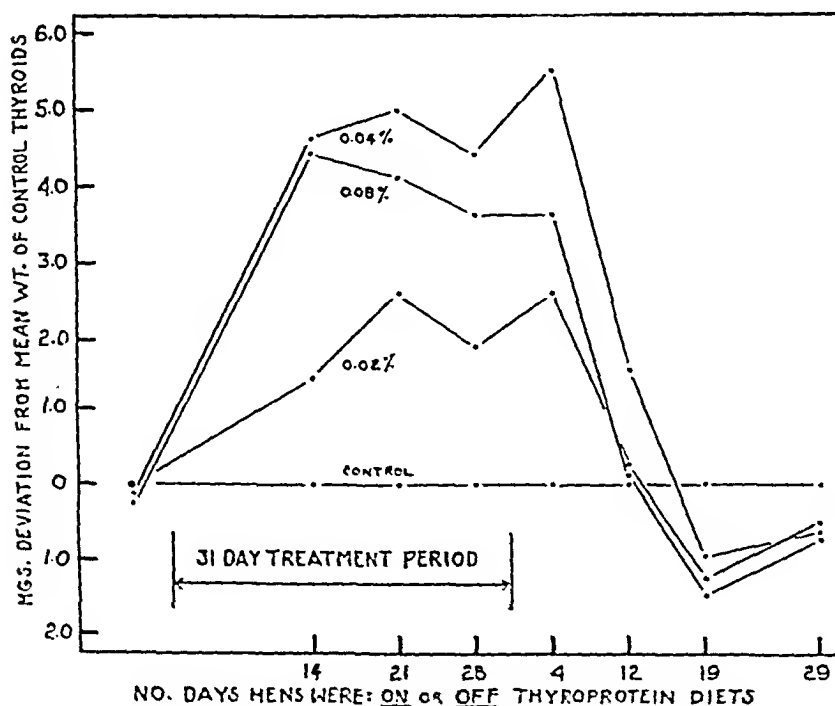


FIG. 2. Goiterogenic Effect in Chicks from Eggs Laid by Hens Fed Quantitative Levels of Synthetic Thyroprotein.

and the degree of goiterogenicity is not proportional beyond the 0.04% level. No greater response was obtained with the 0.08% than with the 0.04% level.

Thyroid enlargement was maximal 14 days after the hens were put on the experimental diets and there were no further increases when treatment was continued for 21 or 28 days. Thyroid weight was not decreased in chicks hatched from eggs laid four days after the experimental diets were discontinued. (This is in contrast to the observations on mean length of incubation period, since by this time the effect of thyroprotein had completely disappeared in chicks from hens fed the 0.02% level.) By the time the hens had been off treatment for 12 days, thyroid weights of their chicks fell within the normal range. However, at the next hatching date (19 days off treatment) there was a consistent sub-normal depression of thyroid weight in chicks from hens that had been treated. The decrease is statistically significant for the 0.02% and 0.08% levels of treatment.

Figure 1 reveals that increase in thyroid weight reaches a maximum level shortly after treatment is initiated and following cessation of treatment there is a residual effect during the first week. This is followed by a sharp depression in thyroid weight below the normal range. Twenty-nine days subsequent to the end of treatment an apparent return to normal thyroid weight was observed.

DISCUSSION

The present experimental results show that hens fed supplemental thyroxine in the form of synthetic thyroprotein produce offspring which: (1) require a longer incubation period, and (2) exhibit marked thyroid enlargement. These two phenomena occur concomitantly and appear to be rather directly associated with each other and with the dosage used except in the instances noted.

One of the instances concerns the response to quantitative dosage. Delayed hatching response appears to be almost directly related to dosage level whereas thyroid enlargement is proportional only for the two lower dosages. The lack of additional goiterogenicity with levels of thyroprotein in excess of 0.04% may be due, however, to inability of the chick thyroid to respond further. Bates, Riddle and Lahr (1941) have demonstrated the existence of thresholds of response of the (newly-hatched) chick thyroid to injected thyrotropin as well as a genetic difference between strains in ability to respond to progressively larger dosages. The work of McCartney and Shaffner (1948) tends to support our view that the maximum goiterogenic response of the chick may have been reached. They obtained chick thyroids 1.5 and 2.75 times heavier than the controls by feeding thiouracil to hens at levels of 0.1% and 0.3% respectively. These values are comparable to the ones obtained in the present study with 0.02% and 0.04% thyroprotein. It may be reasoned that, since thiouracil is an extremely

potent goiterogenic agent, and since they found the higher level approached the maximal level of tolerance of this drug, the goiters produced in the present study were as large as could be produced.

The other exception concerns the time of appearance and of disappearance of the two effects. At the low level of treatment the delayed hatching effect appeared first after treatment started and was less persistent when treatment ceased, than was the goiterogenic effect. However, the absence of these "time" effects at either of the two high dosage levels suggests the differences are of minor significance.

The present experimental data indicate a definite "transfer effect" from hens to their chicks via the egg. Riddle (1930) first advanced the suggestion that maternal thyroid hormone deposited in the egg plays a role in regulating rate of embryonic development and length of incubation period. Riddle (1930) and Hollander and Riddle (1946) observed that pigeons with naturally occurring goiters and symptoms suggestive of myxedema required an extra day or two to hatch. Correction of the dams' hypothyroidism with supplemental iodine corrected both the goiter and delayed hatching of the offspring. Landauer (1942) described a "maternal effect" in which Frizzle mothers apparently failed to deposit in their eggs all the substances essential for normal development. In a previous paper, Aberle and Landauer (1935) showed that day-old homozygous Frizzle chicks had larger thyroids than did the White Leghorns used as controls. Ukita (1919) reported prolonged gestation and thyroid hypertrophy in the young of rabbits thyroidectomized during pregnancy. In all these cases of maternal "transfer effects," the dams were hypothyroid and produced young with enlarged thyroids which seems to substantiate Riddle's hypothesis that maternal thyroid hormone is transferred to the developing embryo. There is a striking similarity between these cases and the data reported here.

Although the mechanisms involved in the production of goiterous offspring which require an increased incubation period are not known, it is possible to postulate at least three explanations. These effects could be due to: (1) reduced deposition of maternal thyroid hormone in the egg; (2) direct transfer of thyroprotein substance(s); (3) a combination of these two factors.

Perhaps the most logical way of explaining the results presented in this paper is to assume that maternal thyroid hormone deposited in the egg influences the size and function of the chick thyroid and length of incubation period. Then, insufficient maternal hormone in the egg might be expected to be reflected in an increased secretion of thyrotropin by the chick pituitary thus causing enlargement of the chick thyroid. The delayed hatching could result from reduced rate of embryonic development or the ineffective functioning of the chick thyroid. Conversely an oversupply of maternal hormone in the egg

might result in thyroid reduction. This is merely an extension of the Moore-Price phenomenon in which excessive maternal thyroid hormone in the egg would inhibit the thyrotropic activity of the chick pituitary and a deficiency of maternal hormone would have the opposite effect.

This postulation is supported by ample evidence that both the thyroid and pituitary of the developing chick are functional prior to hatching. The chick thyroid produces colloid beginning on the 10th day of incubation and in increasing amounts thereafter (Hopkins, 1935). In addition, iodine determinations also indicate increasing thyroid activity from the 10th day to hatching (Sun, 1933). Moreover, amphibian larvae tests indicate the presence of active thyroid hormone in the gland beginning on the 10th day and in quantities on the 18th day (Hopkins, 1935; Uhlenhuth and Ebeling, 1925). Fugo (1940) has shown that the chick thyroid will differentiate in the absence of the pituitary but its functional activity and growth subsequent to the 14th day of incubation are dependent upon hypophyseal stimulation. He did not report that any of his hypophysectomized chicks hatched although one chick lived within the shell for eight days after the control chicks had hatched. It is thus likely that the presence of a functional thyroid controls the mechanism and the time of hatching.

In order to reconcile the present data with the proposed hypothesis it is necessary to assume that the thyroïdal substance (thyroxine) in synthetic thyroprotein is not deposited within the egg in significant quantities. Turner, Irwin, and Reineke (1945) were unable to detect the presence of thyroïdal (or antithyroid) substance in the eggs of thyroprotein-fed hens, and McCartney and Shaffner (1948) were unable to modify chick thyroid weight by injecting crystalline d,l-thyroxine into normal hens. Examination of these data suggests that the thyroactive substance in synthetic thyroprotein differs biologically from natural thyroprotein (thyroid hormone) in not being deposited in the hen's egg.

It is likely that the presence of thyroid hormone or of an effectively functioning chick thyroid may be necessary for the process of hatching (Fugo, 1940). Furthermore, deviations in amount of thyroid hormone production or presence at or near hatching appear to determine whether hatching will be delayed. For instance, Grossowicz (1946) was able to delay hatching proportionately to the amount of thiourea he injected into normal eggs. With the largest dosage the chicks hatched 9 to 10 days late. To return to the present data the tendency toward slightly accelerated hatching and concomitant decrease in chick thyroid weight following the cessation of treatment may be explained as the result of greatly increased maternal thyroid activity. This would be expected to follow the removal of the inhibiting influence of thyroprotein on the hen's pituitary. The data suggest

that removal of inhibition is followed by temporary overproduction of maternal thyroid hormone and a subsequent return to normal thyroid activity. Similar overstimulation of a target organ followed by a gradual return to normal has been noted in this laboratory when pellets of diethylstilbestrol have been implanted subcutaneously in young cockerels. Shortly after the pellet is completely absorbed, the testes increase markedly in size then regress to normal dimensions. There is other evidence in the literature that an oversupply of thyroidal substance in the egg can produce in the chick thyroid the opposite effect of a deficient supply. McCartney and Shaffner (1948) injected d,l-thyroxine into eggs of normal hens and thereby reduced chick thyroid size significantly.

The second hypothesis which cannot be dismissed completely at this time explains the delayed hatching and the goiterogenic phenomena as direct effects of a thyroprotein "factor" transferred to the chick through the egg. The main evidence which might support this view is the recent finding that thyroprotein fed in small quantities (0.002% of the diet) to growing chicks enhances the goiterogenic effect of thiouracil (Moreng and Shaffner, 1948). The hypothesis is untenable at the present time, however, because of the inability to detect the presence of thyroidal (or antithyroid) substances in the eggs of thyroprotein-fed hens (Turner, Irwin, and Reineke, 1945). Moreover, if these effects were due to a deposited thyroprotein factor, neither the tendency toward accelerated hatching nor decreased thyroid size (below normal) would be expected to follow cessation of treatment of the hens. The second hypothesis could not account for these "over-compensations" because the assumption of a direct goiterogenic effect of deposited thyroprotein requires a return to normal activity of the embryonic chick thyroid when the hens are replaced on the basal diet.

By means of a third hypothesis the effects of delayed hatch might be attributed to a deposited thyroprotein, or some substance associated with thyroprotein, and the goiterogenic effect to a deficient maternal thyroid hormone. The evidence upon which this postulation is based concerns the discrepancy between goiterogenicity and delayed hatching at the highest dosage level, and the "time" effects of appearance and disappearance of the two effects at the low level of treatment. However, as has been noted previously, these apparent discrepancies may be accounted for within the first hypothesis by assuming: (1) the chick thyroid is unable to respond further to thyroprotein dosages in excess of 0.04%, (2) the "time" effects to be of minor importance since they do not occur at either of the two higher treatment levels. Therefore, the first hypothesis appears to be the most tenable of the three suggested.

The first hypothesis presumes that increase in thyroid size is the result of uninhibited activity of the chick pituitary. The resulting

secretory products of the stimulated chick thyroid are apparently stored or are ineffective in inhibiting the pituitary. Storage is indicated by the presence in the goiterous thyroids of greatly enlarged, colloid-filled follicles associated with an epithelium of low height (Wheeler and Hoffmann, 1948c). Evidence that the secretory products are relatively ineffective is inferred from the fact that the chicks have lowered oxygen requirements at hatching as tested by the closed-vessel technic (McCartney and Shaffner, 1948). Thus it appears that the chicks with goiters are hypothyroid.

Whether the delayed hatching is a result of the hypothyroidism is not clear at this time. Delayed hatching could be due to: (1) reduced rate of embryonic development as a direct result of deficient maternal hormone, and/or (2) ineffective functioning of the chick thyroid during the last half of the incubation period. Further study is indicated in order to elucidate these points. It should be mentioned however, that preliminary study in this laboratory tends to indicate that rate of early embryonic development (determined by somite counts at 36 hours) is not reduced.

SUMMARY

Offspring of hens fed supplemental thyroxine in the form of synthetic thyroprotein at levels of 0.02%, 0.04%, and 0.08% of the diet require highly significantly longer periods of incubation and exhibit marked thyroid enlargement. The two phenomena occur concomitantly and appear to be rather directly associated with each other and with the dosage employed.

An hypothesis to explain these results and which best fits the present data assumes that maternal thyroid hormone deposited in the egg influences the size and function of the chick thyroid. The results appear to be due to a reduction of maternal thyroid activity and a failure of the thyroidal substance in thyroprotein to be deposited in the egg. The inhibition of the hen's thyroid results in the deposition of insufficient amounts of maternal hormone in the egg which is reflected in enlargement of the chick thyroid and delayed hatching. Release of inhibition of the hen's thyroid when the hen is replaced on the control diet is followed by deposition of an oversupply of maternal hormone in the egg which is reflected in a reduction of the chick thyroid to subnormal size and by a tendency toward accelerated hatching. Evidence is presented which suggests that the secretory products formed by the enlarged chick thyroid as a result of uninhibited pituitary stimulation are stored and are ineffective.

Delayed hatching could be due to: (1) reduced rate of embryonic development as a result of deficient maternal hormone, or (2) ineffective functioning of the chick thyroid during the last half of incubation.

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EFFECT OF ADRENALECTOMY AND ADRENAL CORTICAL HORMONE UPON THE FORMATION OF ANTIBODIES

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ONE OF THE striking characteristics of the adrenalectomized animal and of the Addisonian patient is a well-known susceptibility to infection.

Numerous attempts to study the effect of adrenal insufficiency upon the development of immunity has resulted in conflicting data. Interest in the relation of the adrenal cortical hormones to the development and release of antibody globulin in experimental animals has been revived in recent years by the work of Dougherty, White and associates (1945, 1946). These authors have noted an enhancement of immune titers in animals treated with cortical hormones or adrenotrophic fractions of the hypophysis during the period of immunization. Furthermore, animals previously treated with antigens, and in whom maximum titers had been attained, exhibited a remarkable augmentation of such titers following single injections of adrenal cortical extract or adrenotrophic hypophyseal extracts. This augmentation persisted for a period of 12 hours before returning to pre-injection levels. The anamnestic effect exceeded the increase in immune titers induced by non-specific protein and specific antigen administration. Concurrent reduction in total lymphatic tissue, coupled with the observation that lymphocytes of immunized animals may yield immune globulin, led these authors to conclude that the adrenal hormones effected the dissolution of lymphocytes, thus releasing antibody to the constituent proteins of plasma. The hypothesis is further supported by two observations: (1) that adrenotrophic hormone fails to affect the titratable antibody in adrenalectomized animals; and (2) that desoxycorticosterone alone will neither augment initial titers nor produce the anamnestic effect after titers have been attained.

The present study was designed to yield information on three pertinent questions: (1) the capacity of the adrenalectomized animal to develop and maintain antibodies; (2) the effect of adrenal hormone in the release and/or distribution of antibody protein; (3) the relation-

ship of the peripheral circulating lymphocyte values to the degree of attained immunity.

METHODS

Eleven normal adult cats and 3 normal adult rabbits were employed in these experiments. The animals were acclimatized to the laboratory for a period of at least 6 weeks prior to the experimental period.

The cats received a diet of 100 gm. of "Puss in Boots" cat food and 50 cc. of whole milk daily. The rabbits were fed Purina rabbit pellets with supplements of fresh lettuce every other day. All animals received water ad libitum. Bilateral adrenalectomy was performed in two stages, the operations being separated by a 2-week interval.

The animals were treated with whole adrenal cortical extract for a 10-day recovery period following the second operation. They were then maintained on 1 mg. daily of desoxycorticosterone acetate (DCA) in oil.

The antigen employed was a 10% suspension of fresh washed sheep erythrocytes in normal saline.

For the purpose of immunization, 2 series of antigen injections were administered intravenously. Each series consisted of 5 cc., 3 cc. and 3 cc. of antigen given on consecutive days. One day was allowed to elapse between series.

The hemolysin titration was carried out by the doubling dilution method, employing a 2% suspension of fresh washed sheep erythrocytes as antigen. Intermediate dilutions were made where finer determinations were necessary. Two units of standardized complement derived from pooled fresh guinea pig sera were used in the hemolysin titrations. All dilutions, in separate tubes, were incubated for one-half hour in a water bath at 37.5° C., after which the titers were read. Titers were reread after 12 hours of refrigeration. The highest dilution of antisera producing complete hemolysis of the sheep red blood cells was taken as the end-point of these determinations. Blood counts, including evaluation of RBC, WBC, determination of hemoglobin by the photometer method, and differential counts by the supravital technique, were done at weekly intervals. The serum sodium levels were determined by the method of Butler and Tuthill (1931).

EXPERIMENTAL

Comparison of ability of normal and adrenalectomized cats maintained with desoxycorticosterone to form antibodies. Five cats were adrenalectomized and allowed to recover as described above. In view of the observation of Dougherty, Chase and White (1945) that desoxycorticosterone failed to influence the levels of circulating antibody, these animals were maintained following recovery with 1 mg. of DCA daily throughout the experimental period. With this dosage of DCA the plasma sodium values were maintained at normal levels. Seven days were allowed to elapse following initiation of DCA therapy before immunization was started, in order to allow establishment of an adequate equilibrium. These animals, with 6 intact control cats, were then immunized with a 10% suspension of washed sheep erythrocytes in 2 series as described above. All experimental animals survived this

immunization procedure in good condition. Marked anaphylactic reactions were observed in both the normal and the adrenalectomized cats during the second series of antigen injections. These reactions were characterized by: (1) dilatation of the pupils (2) nystagmus (3) salivation (4) marked slowing and diminished strength of pulse (5) initial acceleration followed by marked slowing of respiration (6) apprehensive cry (7) relaxation of sphincters and mass peristalsis.

Blood was drawn daily following the last administration of antigen for the determination of developing immune titers. Maximum titers were attained on the fifth day following the last injection of antigen.

TABLE 1

Group	Procedure	Animal	Maximum hemolysin titer*
I	Normal Intact	Cat ME	1:320
		Cat NE	1:160
		Cat NI	1:160
		Cat NG	1:80
		Cat NK	1:40
		Cat NL	1:40
		Average	1:133
II	Adrenalectomized (DOCA 1 mg. daily)	Cat NN	1:320
		Cat MN	1:160
		Cat NF	1:40
		Cat NJ	1:40
		Cat NC	1:40
		Average	1:120

* Highest titer developed during the experimental period.

As can be seen in Table 1, the titers attained by adrenalectomized DCA-maintained cats were equal to the highest titer exhibited by the control group. There is no significant difference in the range of titers shown by either group.

Following termination of the entire experimental period, DCA was withheld and all adrenalectomized animals died in acute adrenal insufficiency.

The variation in the leucocyte levels of the normal and adrenalectomized cats throughout the experimental period is illustrated in Figures 1 and 2. Although the fluctuations in all leucocyte values were great throughout the period of observation, neither these nor the absolute values of lymphocytes seemed to be related to the function of the adrenal gland nor to the degree of immunity attained by the animals.

The influence of whole adrenal extract upon the release or distribution of preformed antibodies in intact and adrenalectomized animals. Having established the capacity of the adrenalectomized animal to form antibodies, it was of interest to determine the effect of whole adrenal

Leucocyte Reactions in Normal Immunized Cats.

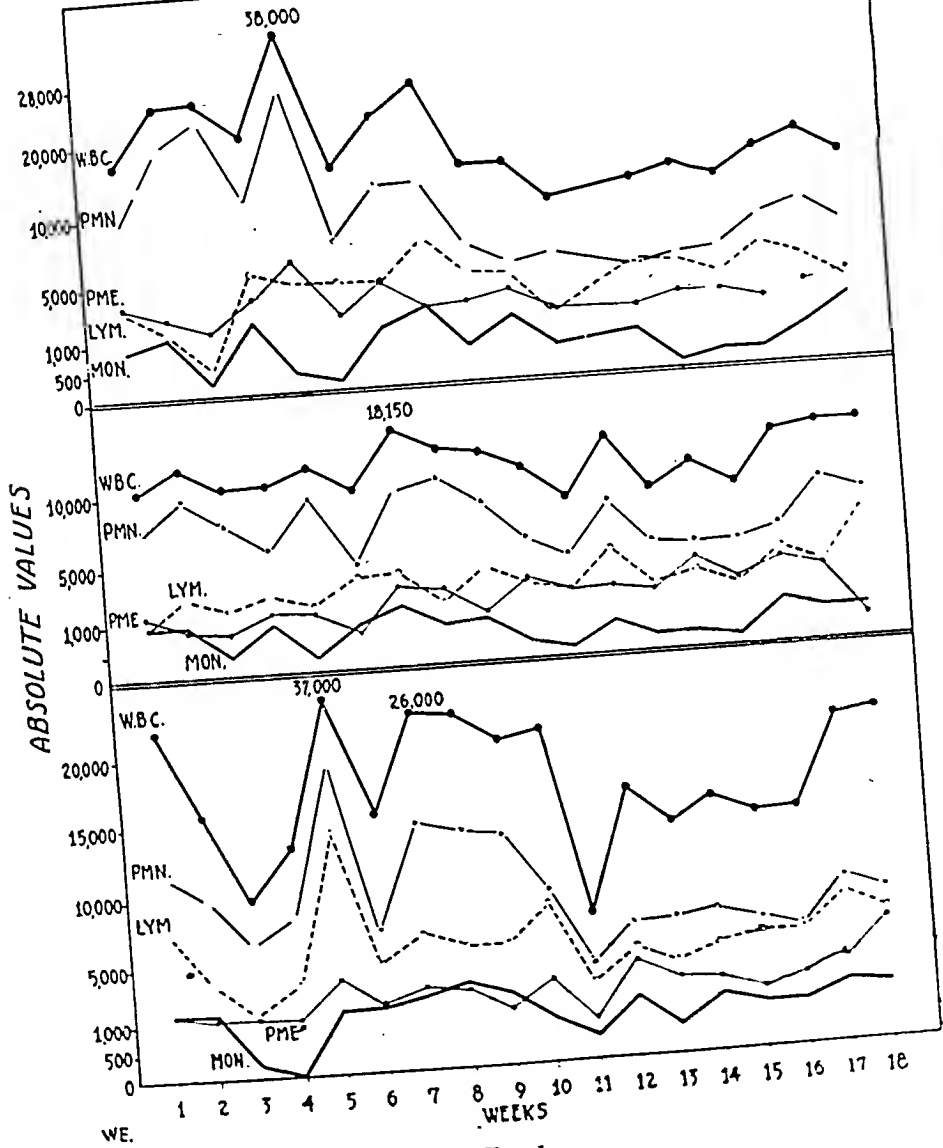


FIG. 1

cortical extract upon the circulating level of these specific immune bodies.

For these experiments, the same 2 groups of animals described above were employed. A single 10 cc. dose of whole adrenal extract (O.S.U.¹) was administered both by the intravenous and subcutaneous

¹ This whole adrenal extract was prepared from whole beef adrenal glands according to the method described by Thatcher and Hartman (1946). Each cc. of extract represented 130 grams of beef adrenal gland tissue.

DISCUSSION

The ability of the animal organism to yield antibodies following adrenalectomy has been the subject of considerable controversy. The literature reveals that in some instances titers were increased, in others decreased (Jaffe and Marine, 1924; Blanchard, 1931). Perla and Marmorston-Gottesman (1929) report temporary augmentation, with subsequent reduction. More recently, Murphy and Sturm have reported marked increases in antibody globulin in adrenalectomized rabbits (1947). Chase, White and Dougherty (1946) have pointed out the difficulties involved in the maintenance of adrenalectomized animals for such study and suggested that accessory cortical tissue or incomplete removal of glands could account for the variations reported. It has been the experience of many workers, however, that cats may be more readily rendered cortically insufficient than most other species of laboratory animals.

The results here indicate that adrenalectomized cats maintained on DCA are capable of forming antibodies in magnitude equal to those of normal intact cats. These findings are in agreement with the results obtained by Eisen and associates using similarly treated rats (1947). It may be argued that since DCA maintains a certain degree of physiological equilibrium it may also sustain the mechanism for antibody formation. However, Dougherty, Chase and White (1945) have emphasized that desoxycorticosterone has no effect upon the antibody levels of intact immunized animals nor does it elicit an anamnestic response in such animals. The fact that all of the adrenalectomized cats in our series died in acute adrenal insufficiency following withdrawal of DCA therapy attests the completeness of their adrenal deprivation. In contrast to the findings of Chase, White and Dougherty (1946), all attempts to produce an anamnestic response with adrenal cortical extract failed. The failure of whole adrenal extract to produce an anamnestic response in the adrenalectomized animals is particularly significant, for if the levels of antibody titers are to be explained by the rate of lymphocyte lysis (White and Dougherty, 1946), whole adrenal extract, acting upon the hyperplastic lymphoid tissue of adrenalectomized animals, would be expected to raise the titers to relatively high levels.

Our inability to reproduce the anamnestic response cannot be attributed to either a species difference or a difference in the type of extract employed, since both cats and rabbits were subjected to trials with 3 different potent extract preparations (O.S.U., Upjohn and Wilson).

Hematologic studies carried out concurrently on normal immunized cats and rabbits and adrenalectomized cats revealed wide variations in total lymphocyte levels which have no relation to the presence or absence of the adrenal gland nor to the capacity of the animal to form or maintain specific hemolysin titers.

SUMMARY

Adrenalectomized cats maintained with DCA demonstrated an ability to produce antibodies at levels comparable with those shown by normal intact control cats. Single large doses of adrenal cortical extract failed to alter titers of immune body when they were established. There was no demonstrable correlation between the levels of circulating lymphocytes and the antibody titers studied.

ACKNOWLEDGMENT

The authors wish to acknowledge the work of Mr. Adolph Ackerman, who carried out the hematologic studies on the experimental animals reported here.

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NOTES AND COMMENTS

ATTEMPTS AT PHARMACOLOGIC BLOCKADE OF THE SECRETION OF ADRENOCORTICOTROPHIN

Recently, Long (1947) has reviewed the evidence in support of the hypothesis that adrenocorticotrophin is released from the hypophysis in response either to circulating epinephrine or to adrenergic nerve stimulation. Markee and others (1947) have advanced a similar theory in connection with post-coital ovulation in rabbits. Sawyer et al. (1947) reported that the pre-treatment of rabbits with Dibenamine prevented ovulation from occurring following the direct instillation of epinephrine into the hypophysis.

In view of the current interest in drugs which block adrenergic responses, it was considered pertinent to test the effects of large doses of Dibenamine and tetraethylammonium chloride on the ability of the pituitary-adrenal axis to respond to a standard test stimulus.

In the first experiment it was found that the intraperitoneal injection of 25 mg./kg. of body weight of Dibenamine itself produced a 35% fall in adrenal ascorbic acid concentration (method of Roe and Kuether, 1943), which then rose steadily to normal levels within ten hours. However, since Nickerson and Goodman (1947) have shown that Dibenamine exerts its

TABLE 1. ADRENAL ASCORBIC ACID CONCENTRATIONS IN "DIBENAMIZED," COLD-EXPOSED AND CONTROL ANIMALS ONE HOUR AFTER INTRAPERITONEAL INJECTION WITH CCl_4 (3ml./kg.) (Means and their standard errors are given for each group)

Group	No of Rats	Description	Body Wt. Grams	Adrenal Wt. mg/100 gm. rat	Adrenal Ascorbic Acid mg./100 gm. adrenals	Adrenal Ascorbic Acid percent decrease
A	6	Controls	170 ± 6.5	15.3 ± 0.78	429.5 ± 22.7	
B	6	CCl_4	167 ± 3.8	15.0 ± 1.51	287.5 ± 15.1	33
C	6	Dibenamine	169 ± 6.2	14.8 ± 0.85	450.6 ± 18.4	
D	5	Dibenamine and CCl_4	165 ± 7.5	12.8 ± 1.10	292.8 ± 7.6	35
E	6	Exposed 7°C.	168 ± 5.0	14.9 ± 0.54	468.5 ± 13.0	
F	5	Exposed 7°C. and CCl_4	164 ± 7.5	16.2 ± 1.46	324.6 ± 22.0	31

blocking action over a period of days, the feasibility of testing ACTH response 24 hours after injection of the drug was considered. Accordingly, in a second experiment six groups of animals were studied as indicated in Table 1. The standard stimulus was 3 ml. of CCl_4 per kg., intraperitoneally, and animals were sacrificed (pentobarbital anesthesia) 60 minutes following this injection. Rats in groups C and D were injected with Dibenamine (25 mg. per kg. of body weight) 24 hours before the carbon tetrachloride test. Since the animals given Dibenamine were known to have had a transitory activation of the ACTH mechanism on the day before the test, another set of rats were included as controls. These were exposed to a temperature of 7° C.

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for three hours one day before carbon tetrachloride injection. The experimental results, summarized in Table 1, show that under the conditions of these experiments Dibenamine does not inhibit the response of the pituitary-adrenal axis to a standard stress.

It should be pointed out that, although a block of the ACTH response in the "Dibenamized" animals would have constituted strong presumptive evidence in favor of the physiologic participation of some epinephrine-like substance in the response, the failure to produce a block does not rule out the possibility that epinephrine may be involved in the release of pituitary ACTH. Nickerson and Goodman (1947) have described certain responses to epinephrine which are not blocked in the Dibenamine-treated animal.

TABLE 2. ADRENAL ASCORBIC ACID RESPONSE TO INTRAPERITONEALLY ADMINISTERED CCl_4 IN TETRAETHYLAMMONIUM-PRIMED RATS AND CONTROLS
(Means and their standard errors are given for each group)

Group	No. of Rats	Description	Body Wt. Grams	Adrenal Wt. mg./100 gm. rat	Adrenal Ascorbic Acid mg./100 gm. Adrenals	Adrenal Ascorbic Acid percent Decrease
A	5	Controls	161 ± 3.9	15.9 ± 1.0	482.6 ± 19.1	
C	6	CCl_4	161 ± 6.8	13.5 ± 0.8	333.8 ± 18.7	31
B	6	TEA	163 ± 6.1	14.3 ± 1.0	448.1 ± 14.8	
D	6	TEA and CCl_4	167 ± 5.8	12.9 ± 0.7	333.5 ± 13.4	26

In a third experiment the ACTH response was tested in animals which had been injected intraperitoneally with 10 mg./kg. of tetraethylammonium chloride (Acheson and Moe, 1946) five minutes before the injection of CCl_4 . Although the injection of tetraethylammonium itself produces no significant fall in adrenal ascorbic acid detectable after 65 minutes, it did not prevent a fall of 26% in animals that received CCl_4 intraperitoneally (See Table 2). Thus, under the conditions of this experiment the chemo-receptor function of the anterior pituitary in the presence of a standard stimulus was unimpaired by pretreatment with tetraethylammonium ion. However, it can not be stated from available evidence that the autonomic ganglia of the tetraethylammonium-injected rats were completely blocked for the duration of the test period.

These results, then, neither support nor invalidate the epinephrine hypothesis of ACTH stimulation. It is relevant to remark, however, that the many patients who have received Dibenamine and/or tetraethylammonium probably have not at the same time experienced a transitory adrenal insufficiency of pharmacologic origin.

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(Aided by a grant from the Hendricks Research Fund of the Syracuse College of Medicine.)

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ERRATA NOTICE

In the text of the paper by Ingle and Nezamis, entitled "The Work Performance of Adrenalectomized Rats Given Continuous Intravenous Infusions of Glucose" which appeared in *Endocrinology* 43: 261-271 (October) 1948, it was erroneously stated that adrenal cortex extract (ACE) was administered in amounts of 20 cc. per 24 hours per rat. The correct amount, as shown in Figures 1 and 2, was 5 cc. diluted to a 20 cc. total volume per 24 hours per rat.

ASSOCIATION NOTICE

ANNOUNCEMENT OF THE 1949 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-First Annual Meeting of The Association for the Study of Internal Secretions will be held in the Chalfonte-Haddon Hall, Friday and Saturday, June 3, and 4, 1949, in Atlantic City, New Jersey.

We are informed by the hotel management that reservations will be difficult to secure on short notice; therefore, members are urged to make reservations at once with Chalfonte-Haddon Hall, giving time of arrival and length of stay in Atlantic City.

The scientific sessions will be held in the Viking Room, as formerly, and registration will be on the same floor. The annual dinner will be held in the Rutland Room, Friday, June 3rd. at 7 P.M., preceded by cocktails in the same room.

Those wishing to present papers, which will be limited to ten minutes, should send title and four copies of an abstract of not more than 200 words, to Doctor J. S. L. Browne, Royal Victoria Hospital, Montreal 2, Canada, not later than March 1, 1949. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program.

Nominations for the Squibb and Ciba Awards and the Ayerst, McKenna and Harrison Fellowship should be made on special application forms, which may be obtained from the Secretary-Treasurer, Doctor Henry H. Turner, 1200 North Walker, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1949.

POSTGRADUATE COURSE IN ENDOCRINOLOGY

A postgraduate course in Endocrinology, sponsored by the Association for the Study of Internal Secretions, will be held at the Skirvin Hotel in Oklahoma City, February 21-26, 1949.

The faculty will consist of outstanding clinical and research endocrinologists of the United States and Canada. The program will consist of clinics and demonstrations and will be a practical one of equal interest to those in general medicine and the specialists.

The fee will be \$100 for the entire course and applications will be accepted in the order received. Applications should be directed to Henry H. Turner, M. D., Secretary-Treasurer, 1200 North Walker, Oklahoma City, Oklahoma.

NOTES ON THE THIRTIETH ANNUAL MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirtieth Annual Meeting was held in the Palmer House, Chicago, Illinois, June 18 and 19, 1948.

Forty-seven papers were presented and forty-four papers were read by title. Total registration was 458, equally divided between members and non-members. Two hundred, twenty-two were present at the dinner at which the presidential address was given by Doctor C. N. H. Long.

Actions of general interest taken by the Council are as follows:

(1) It was the decision of the Council to hold a Postgraduate Assembly in Oklahoma City, February 21-26, 1949, similar to the one which was so well received in Los Angeles in 1948.

(2) The Council approved the indexing and publication of *ENDOCRINOLOGY*, Volumes 1 to 40, inclusive. These will be published by Mr. Charles C. Thomas, in two editions, one including the indices of Volumes 1 to 25, and the other, Volumes 26 to 40.

(3) It was the Council's decision to publish the transactions of the American Goitre Association.

(4) The Council accepted with much regret the resignation of Doctor Earl T. Engle, Chairman of the Publications Committee, and appointed Doctor Warren O. Nelson to this office. Appreciation was expressed to Doctor Engle for his work as chairman of this committee.

(5) The Committee on Registry of Endocrine Pathology was re-appointed to negotiate with the Scientific Director of the American Registry of Pathology regarding the formation of an Endocrine Registry at the Army Institute of Pathology. A sum not to exceed \$750.00 was appropriated for necessary expense for one year.

(6) It was voted to appropriate \$250.00 for 1949 to the National Society for Medical Research.

(7) Due to increasing cost of labor, paper and all printing materials, and the enlargement of the Journals, the dues were increased to \$11.00 per year which includes subscription to either of the Journals, with a combination offer of \$16.00 for both Journals.

(8) The Council voted that the thirty-first Annual Meeting be held June 3 and 4, 1949, in Atlantic City, New Jersey.

(9) A list of the 1948-49 Officers, Council and Committees follows:

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ASSOCIATION AWARDS FOR 1949

THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb & Sons Award of \$1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner; 1941—Dr. Philip E. Smith; 1942—Dr. Fred C. Koch; 1943—no award was given; 1944—Dr. E. A. Doisy; 1945—Dr. E. C. Kendall; 1946—Dr. Carl G. Hartman; 1947—Drs. Carl F. and Gerty T. Cori; 1948—Dr. Fuller Albright. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology:

THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russel; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl G. Heller. The work cited may be either in the field of preclinical or clinical endocrinology. The Award is for \$1,200.00. If within 24 months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, the Award will be increased to \$1,800.00.

THE AYERST, McKENNA & HARRISON FELLOWSHIP

The Ayerst, McKenna & Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin, and in 1948 to Dr. Ernest M. Brown, Jr. This Fellowship is designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. The Fellowship may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend for the Fellowship will vary in accordance with the qualifications of the appointee, but will not exceed \$2,500.00. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. The nominee must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals; submit a program of proposed study; indicate one or more institutions where the proposed program shall be carried out; submit statements of approval from the investigators with whom he proposes to conduct his research.

Each member has the privilege of making one nomination for each award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to endocrinology and by a bibliography of the nominee's most important publications, and reprints, if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 15, 1949.

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